MEDICAL LIBRARY

APR 9 1947

VOL. XXVIII. No. 1] [FEBRUARY, 1947

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

EDITOR
R. W. Scarff

ASSISTANT EDITORS
G. P. Gladstone, N. H. Martin, H. N. Rydon, F. R. Selbie, D. D. Woods

ADVISORY EDITORS
S. P. Bedson, J. R. Marrack, Wilson Smith

with the support of

E. C. Dodds, J. C. Drummond, C. L. Evans, H. W. Florey,
W. E. Gye, H. D. Kay, J. McIntosh, J. W. McLeod,
J. A. Murray, W. J. Tulloch

DIRECTOR
Paul Fildes

LONDON
H. K. LEWIS & Co. Ltd.
136 GOWER ST., W.C.1

CANADA: THE MACMILLAN COMPANY OF CANADA, LTD., BOND STREET, TORONTO

on behalf of

'The British Journal of Experimental Pathology.' Registered Office: 120 Moorgate, London, E.C.2

Annual Subscription, January to December, post free, Two Pounds net
Single Numbers, Seven and Sixpence net

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY.

EDITORIAL NOTICE TO CONTRIBUTORS.

Scope of Papers.—This Journal was founded for the publication of original communications describing the technique and results of experimental researches into the causation, diagnosis and cure of disease in man. Papers upon bacteriological, biochemical, pharmacological, physiological, serological and other subjects are therefore acceptable when bearing directly upon these objects.

Forwarding Papers and Correspondence.—Papers should be forwarded to Professor R. W. Scarff, Middlesex Hospital, London, W. 1.

General Correspondence relating to the conduct of the Journal should be addressed to Sir Paul Fildes, The Lister Institute, Chelsea Bridge Road, London, S.W. 1.

Subscriptions and business correspondence are received by the publishers, Messrs. H. K. Lewis & Co. Ltd., 136, Gower Street, W.C. 1.

Date of Publication.—The Journal is published bi-monthly, in the middle of February, April, June, August, October, and December.

Rapidity of Publication.—All papers accepted for publication will be printed, so far as possible, in the next following issue of the Journal, if received before the middle of the month preceding publication. Long papers, or papers with illustrations, may, however, be delayed if not received earlier.

Proofs.—To avoid delay, proofs will not be submitted to Authors overseas unless they express a desire to see them, or unless the Editors think it necessary. Overseas Authors are therefore particularly reminded of the importance of preparing their manuscripts in such a way that error need not arise.

Reprints.—25 reprints will be supplied free to authors who are registered as personal subscribers to the Journal. Additional copies must be ordered from the Publishers *before the issue is printed*. Prices on application.

Made and printed in Great Britain.

THE BRITISH JOURNAL OF
EXPERIMENTAL PATHOLOGY

VOL. XXVIII FEBRUARY, 1947 NO. 1

THE INFLUENCE OF CHEMICAL CONSTITUTION ON TOXICITY. PART I: A GENERAL SURVEY OF THE ACRIDINE SERIES.

S. D. RUBBO,

School of Bacteriology, University of Melbourne.

Received for publication September 30, 1946.

In view of the increasing interest in the acridine drugs it has become a matter of importance to formulate, if possible, some criteria influencing their toxicity for animals and animal tissues. To this end the toxicity, both systemic and local, of a number of acridine derivatives has been determined. The compounds described herein are identical with those used by Rubbo, Albert and Maxwell (1942), and Albert, Rubbo, Goldacre, Davey and Stone (1945), in their studies relating the influence of chemical constitution to antibacterial activity. In these works, particularly the latter, it was shown that marked antibacterial activity in the acridine series is dependent on adequate ionization of the drug. Furthermore, in the study of the mono-aminoacridines (Rubbo *et al.*, 1942), it appeared that systemic toxicity for mice and antibacterial activity may be influenced by similar chemical properties. A sufficient number of compounds (65) has now been examined to permit the generalization that systemic toxicity is greatest in the highly ionized acridines and least in those which are poorly ionized. To this extent toxicity and antibacterial action are dependent on the same physico-chemical property but, as will be seen, significant departures from this correlation do occur. These departures foster the hope that substances of low toxicity and high antibacterial activity may be developed in this series.

EXPERIMENTAL.

The systemic toxicity of each substance was determined by using mice, from the same stock, of an average age of 9 to 10 weeks and an average weight of 20–22 g. Each mouse was weighed before injection and the dose calculated accordingly. The injection was given by the subcutaneous route over the lateral surface of the thigh. If the volume for injection was greater than 0·8 ml. a divided dose over both thighs was used. Only in exceptional cases of low toxicity and low solubility was it necessary to inject volumes greater than 2 ml. The animals were observed over 3 days and the deaths recorded.

Calculation of the Average Lethal Dose (L.D. 50) was made by plotting the logarithm of the dose in mg. against percentage mortality, and from the curve, which was almost invariably a straight line, the L.D. 50 was arrived at. The curves for some drugs which have been used clinically are shown in Fig. 1.

It will be noted that the curves in Fig. 1 are based on four dose levels chosen to produce no deaths, 100 per cent death and two intermediate levels between these extremes. Similar dose levels were selected for all derivatives and where this was not possible, on account of short supply, the dose range covered the two

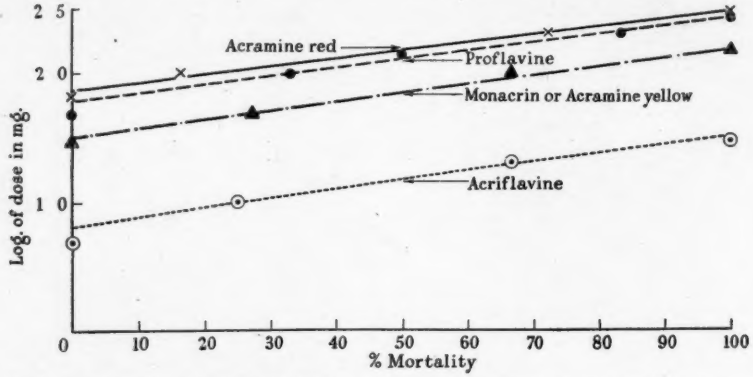


FIG. 1.—Examples of curves obtained in determining Average Lethal Dose (L.D. 50).

extremes and one intermediate dose. As a confirmation of the L.D. 50 values, corrected regression lines for ten compounds were fitted to the transformed dosage-mortality data (Bliss, 1938). From these the logarithm of the L.D. 50 and the standard error of this logarithm were computed. The variation in standard error for these compounds falls between 0·001 g. per kg. body-weight and 0·032 g. per kg. These differences are negligible and are generally applicable to the L.D. 50 values listed in Tables I, II, III and IV.

CORRELATION OF IONIZATION WITH SYSTEMIC TOXICITY.

For the purposes of the following classification a highly ionized acridine is regarded as one possessing a pK_a value equal to or greater than 7·6 (at 20°), that is, at least 70 per cent ionization at pH 7·3 at 20° C. (or 50 per cent ionization at 37° C.). A poorly ionized derivative is one with a pK_a value below this figure.

The results for 36 highly ionized (Nos. 1–36) and 18 poorly ionized acridines (Nos. 37–54) are assembled in Tables I and II respectively. A wide range of chemical types is represented including mono- and diaminoacridines and their derivatives, acridine and acridines not containing amino groups. These are listed in Tables I and II.

From these Tables an average L.D. 50 value and an average anti-streptococcal value might be calculated for the highly ionized and poorly ionized derivatives. These averages unmistakably reveal the tendency for properties of mouse toxicity and antibacterial activity to align themselves with the degree of ionization.

Acridines.	Av. per cent ionization.	Av. L.D. 50 g. per kg.	Av. bacteriostatic dilution (*Strep. Pyogenes*).
Nos. 1–36 (Table I) . .	80 .	0·10 .	1 : 200,000
Nos. 37–54 (Table II) . .	10 .	0·31 .	1 : 12,000

TABLE I.—*Toxicity of Highly Ionized Acridine Derivatives.*

(Compounds in this group are sufficiently ionized to possess marked antibacterial activity.)

No.	Substance.	Number of dose levels tested.	Number of mice used.	L.D. 50 g. per kg. body weight.	Bacteriostatic dilution for *Strep. pyogenes* × 1000.	pK_a in water at 20° C.	Per cent ionized (kation) at 20° C., pH 7·3.
	Aminoacridines.						
*1.	2-Aminoacridine	–	—	0·17	80	8·2	88
*2.	5-Aminoacridine ("Monacrin," "Acramine Yellow")	–	—	0·08	160	9·9	100
3.	2 : 5-Diaminoacridine	5	30	0·14	160	11·4	100
4.	2 : 6- „	4	24	0·11	320	9·2	99
5.	2 : 7- „ ("Acramine Red")	4	58	0·13	160	8·2	88
*6.	2 : 8- „ (proflavine)	–	—	0·14	160	10·0	100
	Substituted 5-Aminoacridines.						
7.	5-Amino-1-methylacridine	5	30	0·13	320	10·2	100
8.	„ 2- „	3	18	0·10	160	10·2	100
9.	„ 3- „	5	30	0·12	160	10·0	100
10.	„ 4- „	4	24	0·07	160	10·0	100
11.	„ 1 : 3-dimethylacridine	4	24	0·10	320	10·5	100
12.	„ 1 : 9- „	4	24	0·04	320	9·3	99
13.	„ 2-methoxyacridine	4	24	0·06	160	10·1	100
14.	„ 3- „	4	24	0·07	320	9·6	100
15.	„ 1-chloroacridine	4	24	0·07	80	8·3	91
16.	„ 2- „	3	18	0·05	160	9·0	98
17.	„ 3- „	5	30	0·11	160	8·8	97
18.	„ 1-hydroxyacridine	4	24	0·07	80	8·3	91
19.	„ 2- „	6	36	0·13	40	7·7	71
20.	„ 3- „	4	24	0·09	160	9·1	98
21.	„ 2-nitroacridine	4	36	0·08	80	7·8	76
22.	„ 1-phenylacridine	4	24	0·07	80	9·2	98
23.	„ 3- „	4	30	0·015	320	9·7	100
24.	„ 3-cyanoacridine	3	18	0·10	80	8·0	83
25.	„ 2-nitro-7-ethoxyacridine	4	24	0·11	640	7·6	60
26.	„ 2-chloro-7-methoxyacridine	4	24	0·11	160	8·6	95
27.	„ 10-methylacridinium hydroxide (as bromide)	5	38	0·14	160	10·6	100
28.	5-Methylaminoacridine	4	24	0·08	160	10·1	100
29.	5-Amino-3-carbomethoxyacridine	3	18	0·14	160	8·5	94
	Substituted Diaminoacridines.						
30.	2 : 8-Diamino-1 : 9-dimethylacridine	4	24	0·09	640	9·0	98
31.	2 : 8-Diamino-3 : 7-dimethylacridine ("Acridine Yellow")	5	54	0·28	160	10·3	100
32.	2 : 8-Diamino-10-methylacridinium hydroxide (as chloride) (purified euflavine or acriflavine)	4	36	0·014	640	12	100
33.	2 : 8-*Bis*-dimethylaminoacridine("Acridine Orange")	4	24	0·25	160	10·8	100
34.	2 : 5-Diamino-7-ethyoxyacridine (" Rivanol ")	4	24	0·12	320	11·5	100
35.	2-Dimethylamino-7-aminoacridine	4	24	0·19	80	8·5	94
	Miscellaneous Acridines.						
36.	2-Amino-10-methylacridinium hydroxide (as bromide)	3	36	0·02	320	12	100

* Rubbo *et al.*

TABLE II.—*Toxicity of Poorly Ionized Acridine Derivatives.*

(Compounds in this group are devoid of marked antibacterial activity because of insufficient ionisation.)

No.	Substance.	Number of dose levels tested.	Number of mice used.	L.D. 50 g. per kg. of body weight.	Bacteriostatic dilution for *Strep. pyogenes* × 1000.	pK_a in water at 20° C.	Per cent ionized (kation) at 20° C., pH 7·3.
37.	Acridine	7	43	0·40	5	4·8	0·3
*38.	1-Aminoacridine	–	—	0·91	5	4·2	0·1
*39.	3- „	–	—	0·33	10	5·8	3·0
*40.	4- „	–	—	0·33	10	6·2	7·4
41.	3 : 7-Diaminoacridine	3	18	0·30	20	6·2	7·4
42.	2 : 9- „	4	24	*ca.* 0·63	20	7·2	44
43.	3-Methoxyacridine	4	19	0·33	10	4·8	0·3
44.	1-Methylacridine	4	12	0·20	<5	4·5	0·2
45.	5- „	3	18	0·21	<5	5·1	0·6
46.	2-Acetamidoacridine	4	24	0·20	10	5·6	2·0
47.	3-Acetamidoacridine	3	18	0·35	10	4·7	0·3
48.	5- „	1	6	>0·30	<5	4·3	0·1
49.	2-Hydroxyacridine	4	24	0·12	10	5·4	1·2
50.	3- „	3	24	*ca.* 0·50	<5	4·7	0·3
51.	2-Amino-7-chloroacridine	2	7	0·14	40	7·0	33
52.	„ 8- „	4	24	0·14	40	7·3	50
53.	3- „ 8- „	3	18	0·22	<5	4·5	0·2
54.	2-Aminoacridine-7-sulphonamide	4	36	0·05	10	7·2	44

* Rubbo *et al.* (1942).

It is evident that while the highly ionized acridines are about three times more toxic than the poorly ionized members, they are approximately sixteen times more active against streptococci. These findings show that ionization must be regarded as a desirable feature for, although it engenders an increase in toxicity for the host, this increase is proportionately far less than the increase in antibacterial activity.

The next point of interest which has a direct bearing on our discussion of ionization is the importance of the various types of ionization. All the compounds so far referred to ionize to varying degrees as *kations*. The insertion of an acidic group, however, would replace this form of ionization by *zwitterionic* and *anionic* ionization, as previously discussed by Albert *et al.* (1945). This change from *kationic ionization* effects a marked reduction in the toxicity of the new derivative compared with the parent molecule. The compounds listed in Table III illustrate this point.

Unfortunately, because of insufficient material, it was impossible to extend these observations to a stage at which there could be no statistical doubt as to the depression of activity effected by anionic ionization. However, in each example of an acridine containing a free acidic group (four examples are given), a very marked reduction in toxicity was noted.

The L.D. 50 of 2-aminoacridine is increased from 0·17 g. per kg. body weight to 0·51 g. per kg. by the insertion of a sulphonic acid group. If the kationic ionization of the latter (No. 55) is restored by forming the amide the toxicity is regenerated to a surprising degree, namely, L.D. 50 of 0·05 g. per kg. body weight (No. 54). Although the abnormally high toxicity of 2-aminoacridine-7-sulphonamide may require further explanation, the difference between this amide and its

TABLE III.—*Toxicity of Acridine Derivatives Devoid of Antibacterial Activity because of Zwitterion Structure.*

No.	Substance.	Number of dose levels tested.	Number of mice used.	L.D. 50 g. per kg. of body weight.	Bacteriostatic dilution for *Strep. pyogenes* × 1000.	pK_a in water at 20° C.	Per cent ionized (kation) at 20° C., pH 7·3.
(1)	2-Aminoacridine (for comparison)	–	—	0·17	80	8·2	88
(54)	2-Aminoacridine-7-sulphonamide (for comparison)	–	—	0·05	10	7·2	44
55.	2-Aminoacridine-7-sulphonic acid	7	42	0·51	<5	7·6	0·0
(2)	5-Aminoacridine (for comparison)	–	—	0·08	160	9·9	100
56.	5-Aminoacridine-3-carboxylic acid	5	30	0·16	<5	9·9	0·0
(38)	Acridine (for comparison)	–	—	0·40	5	4·8	0·3
57.	Acridine-3-carboxylic acid	3	18	>0·60	<5	5·2	0·0
58.	5-Carbomethoxyacridine	3	18	0·24	<5	3·5	0·0
59.	Acridine-3-sulphonic acid	1	6	>0·60	<5	4·7	0·0

corresponding acid emphasizes the significant role of kationic ionization. Similarly, the carboxylic and sulphonic acid derivatives of acridine are so very non-toxic that it is impossible to estimate the L.D. 50 by subcutaneous injection where the volume administered cannot exceed 4 to 5 mls.

It has been shown by Albert *et al.* (1945) that substituents influence antibacterial activity in the general direction predictable by changes in ionization. From the evidence so far presented this statement might also be applied to systemic toxicity, but with the proviso that substituents not affecting ionization may nevertheless play an additional role. The influence of different substituents will now be briefly reviewed.

Amino Derivatives.

The unsubstituted mono- and diamino-acridines fall into two main groups, depending on their degree of ionization. Those which have pK_a value of 7·6 or more have, without exception, an L.D. 50 less than 0·2 g. per kg. body weight (Nos. 1–6 inc., Table I), whilst those with pK_a values less than 7·6 have L.D. 50 greater than 0·3 g. per kg. (Nos. 38–42 inc., Table II). Amino groups in the 2- or 5- positions increase the toxicity by virtue of the ionization which they induce.

Some of these findings do not confirm those of Albert, Dyer and Linnell (1937), who, because of the limited numbers of mice available, were forced to calculate an approximate L.D. 50 dose by using Kärber's formula. By these means they established that the L.D. 50 fell within the range of 0·75 down to 0·05 g. per mouse kg. for a series of 16 compounds tested. In the present series of 65 compounds the range is found to lie between 0·9 and 0·01 g. per mouse kg. Thus it is considered that their conclusion on the detoxifying effect of an amino group in the 2- position must be revised. Furthermore, it is appropriate to point out that 2 : 7-diaminoacridine ("Acramine Red") is no less toxic than proflavine, since the findings of these authors give the impression that its toxicity is only two-fifths that of proflavine (see Fig. 1).

Methyl Derivatives.

Among the methyl derivatives of highly ionized acridines those having a methyl group attached to a carbon atom tend to have a reduced toxicity. This is well demonstrated in the 1-methyl derivative of 5-aminoacridine, a substance of very marked antibacterial activity (No. 7, Table I) and in the 3 : 7-dimethyl derivative of proflavine ("Acridine Yellow," No. 31, Table I), which is significantly less toxic than the parent substance, proflavine. On the other hand, attachment of a methyl group to the ring nitrogen influences toxicity most adversely in the cases of 2- and 2 : 8-diaminoacridines where the ring nitrogen becomes quaternary. However, 5-aminoacridine is not affected, doubtless because its methylation product is not a true quaternary compound (Albert and Goldacre, 1943). It is worth stressing that purified acriflavine (No. 32, Table I) is an extremely toxic drug whose continued use clinically can no longer be justified on this and other grounds.

In the group of poorly ionized acridines only two methyl derivatives, namely, 1- and 5-methyl acridines, were available in sufficient quantity to determine the L.D. 50. These substances (Nos. 44 and 45, Table II), were significantly more toxic than acridine (No. 37).

Miscellaneous Derivatives.

The influence of the remainder of the substituents on toxicity is too variable to warrant any detailed analysis. In most instances methoxy, hydroxy, phenyl, nitro, chloro, or cyano groups do not affect the toxicity. In isolated examples it is interesting to note that a carbomethoxy group in the 3-position effected a significant reduction and a phenyl group in the same position a significant increase in toxicity of 5-aminoacridine (see Nos. 2, 23, 29, Table I).

In 1945 we used the term " dimensional factor " to describe hindrance to adsorption of the derivative on bacterial enzymes, and hence partial suppression of its antibacterial action. It is of some interest to note that the substituent groups causing this dimensional factor, instead of hindering specific adsorption at vital centres in the mammal, appear to facilitate adsorption, since such derivatives are more toxic than the parent substances. These findings are shown in Table IV. On the other hand, mepacrine (No. 65, Table IV), which is somewhat

TABLE IV.—*Toxicity of Highly Ionized Acridines whose Antibacterial Activity is Lessened because of Dimensional Factors.*

No.	Substance.	Number of dose levels tested.	Number of mice used.	L.D. 50 g. per kg. of body weight.	Bacteriostatic dilution for *Strep. pyogenes* × 1000.	pK_a in water at 20° C.	Per cent ionized (kation) at 20° C., pH 7·3.
60.	5-*Cyclo*Hexylaminoacridine	4	24	0·025	80	9·2	99
61.	5-Heptylaminoacridine	4	24	0·05	40	9·7	100
62.	5-Amino-6 : 7 : 8 : 9-tetrahydroacridine	4	24	0·025	5	9·9	100
63.	2-Amino-5-*p*-aminophenylacridine (phosphine)	5	30	0·05	40	8·2	88
64.	2 : 8-Diamino-3 : 7-dimethyl-5-phenylacridine (benzoflavine)	5	30	0·03	80	9·6	100
65.	2-Chloro-7-methoxy-5-(1′-methyl-4′-dimethylaminobutyl)-aminoacridine (*Atebrin* or *Mepacrine*)	5	40	0·28	20	10·2	100

sterically hindered, is significantly less toxic and less active against bacteria than 5-amino-2-chloro-7-methoxyacridine (No. 26, Table I), i.e. the same compound without the side chain.

In the above findings we see a distinction in the response of mammalian cells and bacterial cells to the highly ionized molecules of this series. Since many of the drugs in Table IV have a high oil-water partition coefficient, it might be suggested that their main site of action is in the lipoid phase of the mammalian cell in contrast to their site of action in the bacterial cell, which is probably in the aqueous phase. These results confirm our previous conclusion (1945) that the introduction of dimensional factors reduces the chemotherapeutic value by increasing toxicity and lowering antibacterial activity.

LOCAL TOXICITY OF ACRIDINES ON HEALTHY TISSUES.

Since the report of Mitchell and Buttle (1942) describing the application of proflavine powder to infected gunshot wounds, a number of papers dealing with the local toxicity of aminoacridines have been published. Russell and Falconer (1943) concluded that proflavine, 5-aminoacridine and 2 : 7-diaminoacridine in powder form were highly destructive not only to the brain but also to muscle and other soft tissues. Hawking (1943) came to a similar conclusion, using various salts of proflavine. Selbie and McIntosh (1943), on the other hand, considered that a mixture of proflavine powder, 1 per cent, and microcrystalline sulphathiazole, 99 per cent, had no delaying effect on the healing of aseptic incisions. The conclusion to be drawn from these various studies appears to be that proflavine sulphate or its base induces local necrosis when applied as a powder, but that in 99 parts of sulphonamide this reaction is mitigated.

It was considered worth while in the present studies to extend these observations to poorly ionized acridine derivatives since, without exception, only highly ionized drugs had been used previously. To this end the effect on muscle tissue of 16 derivatives was assessed, using the following implantation technique :

Tablets weighing 5 to 10 mg. were prepared in a pharmaceutical tabletting machine without the addition of any excipient. A tablet of the drug was then implanted into the *vastus lateralis* muscle of an anaesthetized guinea-pig. This muscle was exposed by making a small incision over the lateral surface of the thigh, gently separating fascial sheaths and incising the muscle belly along the line of the fibres. Small dissecting forceps were used to retract the muscle bundles between which the tablet was placed. The wound was closed by interrupted horse-hair sutures. In each case both hind limbs were used, one receiving a tablet of the acridine and the other a tablet of sulphanilamide as a control for tissue damage. In other experiments glass beads were also used as foreign body controls. After three days the animals were killed, the treated muscles removed, prepared for sectioning and stained by haematoxylin-eosin.

The following compounds were tested :

Highly ionized group.—5-aminoacridine, base and hydrochloride ; 5-amino-2-chloroacridine, base ; 5-amino-2-nitro-7-ethoxyacridine, base ; 2 : 8-diaminoacridine (proflavine), base, sulphate, salicylate, and benzenesulphonate ; 2 : 8-diaminoacridine-10-methylacridinium chloride (euflavine) ; 2 : 7-diaminoacridine, base and hydrochloride.

Poorly ionized group.—Acridine hydrochloride ; 3-aminoacridine, base ; 2-hydroxyacridine, base ; 5-hydroxyacridine, base ; 5-acetamidoacridine, base.

Control drug.—Sulphanilamide.

The results can be very briefly summarized by stating that all the acridine derivatives used stimulated practically identical pathological changes in the healthy muscle tissue. In every instance the muscle fibres were swollen and pale-staining with loss of cross striations for a distance of at least 1 cm. from the site of the tablet. In the immediate proximity of the drug necrosis was evident, with marked inflammatory exudate packed with polymorphonuclear leucocytes, mostly intact and showing some evidence of phagocytosis ; a few lymphocytes and young fibroblasts were also present. Slight capillary haemorrhages were noted in some cases.

Sulphanilamide and glass beads did not excite any necrotic changes and little or no inflammatory response was observed.

The above findings fully confirm those of previous authors. They also show that the necrotizing action of acridine drugs is in no way related to their degree of ionization or to the acidity of various salts. As this local toxic action appears to be a common feature of all types of acridine derivatives, little hope can be entertained of modifying this attribute by substitution. Although we conclude that the clinical use of aminoacridines in pure powder is contra-indicated, there appears to be no reason why they should not be applied in a suitable inert diluent as recommended by Selbie and McIntosh (1943). Personal experience in the treatment of wounds with 1 per cent 5-aminoacridine (" Monacrin ") in sulphanilamide powder confirms this belief.

DISCUSSION.

The toxicity of acridine derivatives has been determined at various times by various authors, and these results are summarized in Table V for comparison with the present findings.

It will be noted that in most instances there is little ground for comparison, since different routes of injection have been used, as well as different animal species. Moreover, no correction has been made for varying amounts of water of crystallization and for different acid radicles in the acridine salts. We have attempted to avoid this by adopting a standard technique of injection, using the mouse as our experimental animal, and by expressing the L.D. 50 dose as the weight of the free base per kg. of body weight required to kill 50 per cent of the animals injected.

Any attempt to correlate the chemical constitution of a drug with its toxicity for the living mammal is beset with peculiar difficulties. The lethal action of a drug is the end-point of a chain of events leading to the irreversible poisoning of a group of cells essential to life. Some of the factors involved in this pathway of toxicity are : The solubility of the drug in tissue fluids, the absorption from the site of injection, the transport to susceptible cells, the fixation on the cell surface or penetration into the cell, the rate of excretion, and the chemical detoxification in special organs. The chemical structure of a substance may modify any of these factors and, furthermore, it may modify them in different directions, facilitating one and hindering another. Hence, any conclusion drawn must be conceived on the broadest possible basis, and where possible from a fundamental physico-chemical point of view.

In this study of acridine derivatives it has been possible to select one chemical attribute, namely, the degree of ionization, and to assess the influence of this

TABLE V.—*Toxicity Obtained by Other Authors for Acridine and Some Clinically Interesting Derivatives.*

Substance.	Reference.	Animal and route of injection.	L.D. 50 g. per kg. of body weight.	L.D. 50 present study Mouse S.C.
Acridine	1	Dog i/v	0·09	0·40
	2	Rabbit, i/v	0·10	
	3	Mouse, s/c	0·30	
Acriflavine	4	Rabbit, i/v	0·04	0·02
	5	Mouse, s/c	0·05	
	2	Mouse, i/v	0·04	
	6	Rabbit, i/v	0·03	
	6	Dog, i/v	0·03	
	7	Rabbit, i/v	0·04	
	3	Mouse, s/c	0·05	
	8	Mouse, i/p	0·06	
Proflavine	5	Mouse, s/c	0·25	0·14
	3	Mouse, s/c	0·20	
Rivanol	7	Rabbit, i/v	0·05	0·12
2-7 : Diaminoacridine	3	Mouse, s/c	0·50	0·13
	8	Mouse, i/p	0·30	
5-Aminoacridine	8	Mouse, i/p	0·07	0·08
Mepacrine	9	Mouse, ?	0·21	0·28

1. Jodlbauer (1905) *Arch. Pharmacodyn. Ther.*, **15**, 223.
2. Dyson, 'The Chemistry of Chemotherapy,' 1928.
3. Albert, Dyer and Linnell (1937) *Quart. J. Pharm.*, **10**, 649.
4. Lenz (1921) *Z. ges. exp. Med.*, **12**, 195.
5. Robertson (1925) *Aust. J. exp. Biol. med. Sci.* **2**, 21.
6. Heathcote and Urquhart (1930) *J. Pharmacol.*, **38**, 145.
7. Levrat and Morelon (1933) *Bull. Sci. pharmacol.*, **40**, 582.
8. Ungar and Robinson (1944) *J. Pharmacol.*, **80**, 217.
9. Basu and Box (1941) *Ann. Biochem. exp. Med.*, **1**, 317.

property on the toxicity of the drug. While this is undoubtedly an over-simplification of our problem, there is, with very few exceptions, a good correlation between ionization and toxicity. In general the highly ionized acridines are at least three times more toxic then those whose ionization is poor. The degree of ionization which must be reached in the animal body for this heightened toxicity to be manifest is approximately 50 per cent; that is, any acridine having a pK_a value greater than 7·6 (20° C.) will most probably have an L.D. 50 somewhat less than 0·20 g. per kg. of body weight for mice by subcutaneous injection. Conversely, any acridine having a pK_a less than 7·6 will probably have an L.D. 50 greater than 0·20 g. per kg. In this respect there exists a positive correlation between antibacterial activity and systemic toxicity.

The scatter diagram (Fig. 2), made by plotting the log of the L.D. 50 against the pK_a, shows the same grouping of compounds as in a similar diagram illustrating the connection between ionization and antibacterial activity (Albert *et al.*, 1945).

In further support of this correlation it has been shown that the introduction of acidic groups which replace normal kationic ionization by zwitterionic or anionic forms is accompanied by a marked loss of antibacterial activity and a marked depression of toxicity. Thus it is apparent that this physico-chemical attribute influences these two biological systems in the same direction. However, it is worth noting that the increase in toxicity induced by ionization is proportionately far less than the increase in antibacterial activity, so that the change in the therapeutic index is a favourable one. On these grounds it might be pre-

dicted that all future acridines of clinical value will be highly ionized drugs. Actually, all the acridines now in use clinically fall into this category.

In regard to further substitution not influencing ionization, it is not yet possible to formulate any criteria with which to predict toxicity of a new derivative.

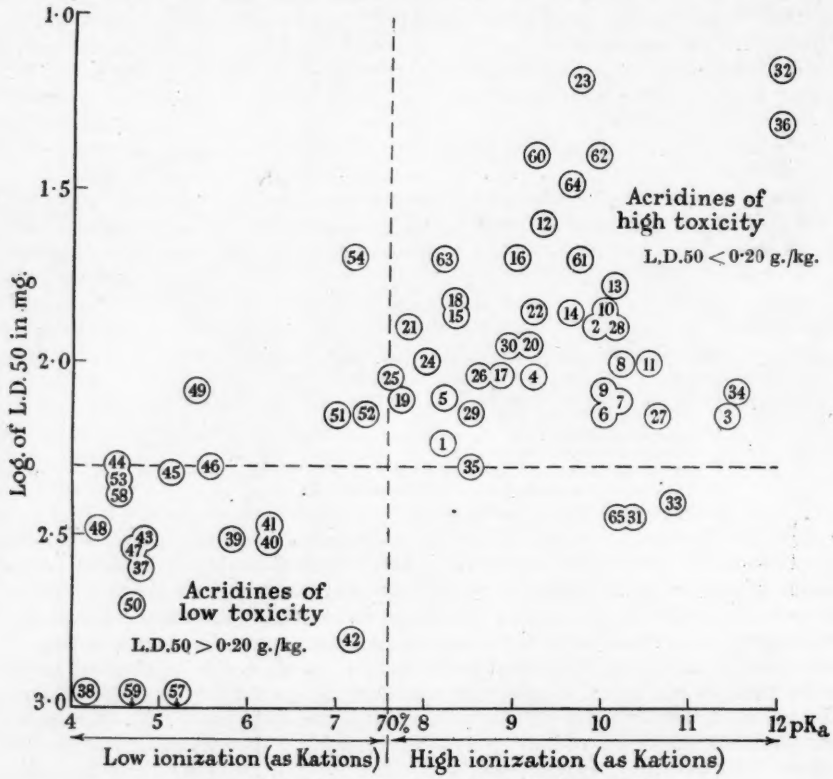


FIG. 2.—The relationship of toxicity to basic strength.

It is legitimate to suppose that the kation has less capacity to penetrate cells and would be fixed at the cell surface, whilst the unionized molecule probably diffuses freely into and out of the cell. Thus it might be suggested that the acridine kations act on tissue cells distinct from those affected by undissociated molecules, or if they react with the same cells they may combine at different receptor sites. For instance, there is a very striking difference in symptoms due to 5-aminoacridine poisoning and acridine poisoning. The former, which is highly ionized, induces very marked hyper-excitability with violent convulsions preceding death, whereas acridine, a poorly ionized substance, exerts a narcotic action with coma preceding death. Thus, it is quite plain that within the acridine series wide differences in pharmacological activity may be detected, in spite of the fact that they may appear, in the chemical sense, to be a related series. It is not

surprising, therefore, that the same substituents should affect toxicity in different directions. However, as already mentioned, the introduction of " dimensional factors " into the molecule (for fuller description see Albert *et al.*, 1945) leads to a lowering of antibacterial activity and to an increase in mammalian toxicity.

Determination of the toxicity of various acridines for healthy muscle tissue failed to reveal any differences associated either with ionization, acidity or substitution. All of the 16 compounds selected for study caused a marked necrosis with inflammation at the site of implantation of the drug.

In conclusion, it is felt that these results might have a broad pharmacological application in that the systemic toxicity of many basic drugs might well be attributed to their kationic ionization. One need only compare well-known examples, such as histamine, a toxic amine having pK_a of 9·91 at 20° C., with histidine, the corresponding non-toxic zwitterion; also tyramine (pK_a 9·3 at 20° C.) with tyrosine. On the basis of the present findings it might be suggested that these amino acids are devoid of toxicity mainly because of their zwitterionic character.

SUMMARY.

1. The systemic toxicity (L.D. 50) of 65 acridine derivatives in mice and the local toxicity of 16 derivatives in guinea-pigs have been determined.

2. In general, kationic ionization increases systemic toxicity in a manner similar to its effect on antibacterial activity, but to a lesser extent. Acridines having a pK_a greater than 7·6 are approximately three times more toxic than the poorly ionized members whose pK_a is below 7·6. The former have about 16 times greater antibacterial activity than the latter. Thus, toxicity and antibacterial activity are dependent on the same physico-chemical property, namely, kationic ionization.

3. It can be predicted that acridines of clinical usefulness will be highly ionized drugs, since the increase of antibacterial action following ionization is proportionately far greater than the increase in systemic toxicity.

4. Local toxic action of acridines in powder form on healthy guinea-pig muscle is severe, and is uninfluenced by the degree of ionization.

5. It is suggested as an extension of the present theory that the toxicity of certain naturally occurring amines (viz. histamine, tyramine, etc.) is dependent *inter alia* on their degree of kationic ionization.

The author wishes to thank his colleague, Dr. Adrien Albert, for making available the drugs used in this study and for his generous help and criticism. Also his thanks are due to Miss Burvill, B.Sc., for assistance in some of the work.

REFERENCES.

Albert, A., Dyer, F. J., and Linnell, W. H.—(1937) *Quart. J. Pharm.*, **10**, 649.
Idem and Goldacre, R.—(1943) *J. chem. Soc.*, 454.
Idem, Rubbo, S. D., Goldacre, R., Davey, M. E., and Stone, J.—(1945) *Brit. J. exp. Path.*, **26**, 160.
Bliss, C. I.—(1938) *Quart. J. Pharm.*, **11**, 192.
Hawking, F.—(1943) *Lancet*, i, 710.
Mitchell, G. A. G., and Buttle, G. A. H.—(1942) *Ibid.*, ii, 416.
Rubbo, S. D., Albert, A., and Maxwell, M.—(1942) *Brit. J. exp. Path.*, **23**, 69.
Russell, D. S., and Falconer, M. A.—(1943) *Lancet*, i, 580.
Selbie, F. R., and McIntosh, J.—(1943) *J. Path. Bact.*, **55**, 477.

THE PRECISION OF PLASMA VOLUME DETERMINATIONS BY THE EVANS BLUE METHOD.

K. MATHER, R. G. BOWLER, A. C. CROOKE AND C. J. O. R. MORRIS.

From The John Innes Horticultural Institution, Merton Park, London, S.W. 19, and the Medical Unit, The London Hospital, Whitechapel Road, London, E. 1.

Received for publication October 14, 1946.

IN essence the Evans Blue method involves the injection of a known quantity of dye into the circulatory system, and the estimation of the concentration of the dye in a sample of plasma taken after mixing has been completed. In practice, however, the concentration of dye is not stable even after the completion of mixing, which occurs in a few minutes; for dye gradually passes out of the plasma. The problem, therefore, becomes one of finding the relation between dye concentration and time by repeated sampling after injection, in order that the concentration can be found at zero time by extrapolation. Knowing the deviations of the sample concentrations from those expected on the basis of the calculated concentration–time relation, the standard deviation of the estimated concentration at zero time can then be found.

The Relation between Concentration and Time.

Five men were injected with Evans Blue and duplicate determinations of the dye concentration made 15, 30, 45, 60, 75 and 90 minutes after injection, using Morris's (1944) chromatographic technique. The results (Table I) were subjected

TABLE I.—*Evans Blue Concentrations in mg. Dye/100 ml. Plasma.*

Time in minutes after injection.	Individual 1.	2.	3.	4.	5.
15	0·738 0·810	0·998 1·020	0·838 0·797	0·797 0·838	0·777 0·743
30	0·745 0·798	0·979 0·959	0·858 0·858	0·831 0·797	0·777 0·797
45	0·818 0·798	0·998 0·979	0·818 0·818	0·797 0·797	0·744 0·723
60	0·798 0·778	0·972 0·950	0·770 0·744	0·723 0·737	0·717 0·763
75	0·798 0·757	1·020 1·080	0·774 0·716	0·697 0·723	0·704 0·743
90	0·778 0·810	0·917 0·939	0·723 0·703	0·723 0·723	0·797 0·723

to an analysis of variance (see Mather, 1946, Section 24). The 59 independent comparisons (degrees of freedom as they are called statistically), which can be made between the 60 observations, are subdivided into 4 groups of different meanings for our purpose. First, we may recognize 4 degrees of freedom between the 5 values obtained by summing all 10 determinations made on each individual. Second, there are 5 degrees of freedom between the 6 totals obtained by summing all the observations made at one time after injection. The third group of 20 degrees of freedom is obtained in a somewhat more complex way. If duplicate determinations are summed we obtain a table of 30 entries, and so containing 29 degrees of freedom. Of these, 9 are recognizable as those already picked out

as resting on differences between Individuals and Times. The remaining 20 form our third group, and represent the Individual-Time interaction, i.e. they are concerned with the variation in the Individual differences over Time, or, if preferred, with variation in the Time differences over Individuals. The fourth group, of 30 degrees of freedom, comes from the differences between duplicate observations, and so is concerned with variations or error in the technique of determining concentration.

The corresponding analysis of the sum of squares of differences between the observations is made as follows. The sum of squares of all 60 individual observations is 40·261465. From this is subtracted a correction term, found as the ratio of the square of the sum of all observations to the number of observations, i.e. $48{\cdot}815^2/80$ or 39·715070. The difference, 0·546393, is the sum of squares of deviations from the general mean and corresponds to the total of 59 degrees of freedom. Next, duplicate observations are pooled to give a 5×6 table (Table I*a*), whose lower margin shows the Individuals totals and upright margin the

TABLE I*a*.—*Table Reduced by Summing Duplicate Observations.*

Time.	Individual.	1.	2.	3.	4.	5.	Total.
	15	1·548	2·018	1·635	1·635	1·520	8·356
	30	1·543	1·938	1·716	1·628	1·574	8·399
	45	1·616	1·977	1·636	1·594	1·467	8·290
	60	1·576	1·922	1·514	1·460	1·480	7·952
	75	1·555	2·100	1·460	1·420	1·447	7·982
	90	1·588	1·856	1·426	1·446	1·520	7·836
Total		9·426	11·811	9·387	9·183	9·008	48·815

Times totals. The 30 entries in the body of the table are squared, summed, and the total divided by two, since each entry is itself the sum of two observations. From the result, 40·243628, is subtracted the correction term, previously found, to leave a sum of squares 0·528557 corresponding to the pooled 29 degrees of freedom for Individuals, Times and Individuals-Times interaction. The sum of squares for the 30 degrees of freedom between Duplicates is found as the difference between those obtained from the full and reduced tables, viz. as $0{\cdot}546393 - 0{\cdot}528557 = 0{\cdot}017836$.

The sum of squares for 29 degree of freedom is next broken up into the three parts for Individuals, Times and their interaction. The first is obtained from the lower marginal totals, which are squared, summed, and the sum divided by 12 to give 40·161377. Subtraction of the correction term leaves 0·446306 for the sum of squares between Individuals. The Times sum of squares is found similarly from the upright marginal totals. Squaring, summing, dividing by 10 and subtracting the correction term gives 0·028886 for this item. The interaction sum of squares is finally obtained as the residual of the sum of squares for the 29 degrees of freedom when the Individuals and Times sums have been deducted, i.e. as $0{\cdot}528557 - 0{\cdot}446306 - 0{\cdot}028886$ or 0·053365.

The analysis of variance may now be set out as in Table II. Dividing the sum of squares for each item of variation by the corresponding number of degrees of freedom gives the mean square or estimated variance which that item contributes. The Duplicates item, measuring errors of chemical estimation, gives much the smallest mean square. Dividing it into the other mean squares, three

TABLE II.—*Analysis of Variance of Data from Table I.*

Item.	Sum of squares.	Degrees of freedom.	Mean square.	Variance ratio.*	P.
Individuals (I.)	0·446306	4	0·111576	187·68	v. small
Times (T.)	0·028886	5	0·005777	9·72	v. small
Interaction (I. × T.)	0·053365	20	0·002668	4·49	0·001
Duplicates	0·017836	30	0·000595	—	—
Total	0·546393	59			

* Using the Duplicates mean square as the estimate of error.

Variance Ratios are obtained whose significance may be found from a table of Variance Ratios such as that given by Mather (1946).

The probability of obtaining a variance ratio as large or larger by chance is in no case so high as 0·001. We must judge that differences between individuals, those between times and also their interaction are significantly larger than can be accounted for by the chemical error, which may therefore be neglected by comparison with the biological variation.

The Individuals-Times interaction mean square is our estimate of the biological error, and against it we may assess whether the Individuals and Times items are larger than would be expected as mere expressions of this error variation. For this purpose we divide the interaction mean square into the other two to give two new variance ratios. That for Individuals has a probability very much less than 0·001, and so the Individuals must still be regarded as differing in their mean concentrations. That for Times is, however, no longer significant, having a probability of over 0·05.

A further analysis of the Times items is, however, possible. The relation of the six times totals to the time intervals can be completely described by a quintic curve. The 5 degrees of freedom for Times can be ascribed one to each of the five successive powers used in building up such a curve (Mather, 1946, Section 36). Of these, however, only the first power, representing a straight line relation between concentration and time, need concern us. Let us consider the best fitting straight line relation $Y = \bar{y} + b(x - \bar{x})$, where Y is the concentration expected at time x, $\bar{y}$ is the general mean concentration at mean time $\bar{x}$, and b is the slope of the line, to be estimated from the data. For ease of calculation x will be measured in units of 15 minutes from injection. The mean of $\bar{x}$ is then $1/6\ (1 + 2 + 3 + 4 + 5 + 6) = 3{\cdot}5$ and the sum of squares of deviations of x from its mean is $10(1^2 + 2^2 + 3^2 + 4^2 + 5^2 + 6^2) - \dfrac{10(1 + 2 + 3 + 4 + 5 + 6)^2}{6}$ or $10(91 - 73{\cdot}5) = 175$. The factor 10 appears because 10 observations were made at each sampling time. The sum of cross-products of concentration and time is found from the Times totals as $(8{\cdot}356 \times 1) + (8{\cdot}399 \times 2)\ \ldots\ + (7{\cdot}836 \times 6) - 1/6\ (48{\cdot}815 \times 21)$, the correction term being the product of the sum of all concentrations and the sum of all times divided by the number of times used. This sum of cross-products reduces to $-\ 2{\cdot}0945$. The slope of the line relating concentration to time is then $b = -\dfrac{2{\cdot}0945}{175} = -\ 0{\cdot}011968$ mg. dye/100 ml. serum per 15 minutes. This line accounts for a portion of the sum of squares between Times totals corresponding to 1 degree of freedom, the remainder for

4 degrees of freedom being due to deviation from the line. The former portion of the sum of squares is found as $\frac{(-2{\cdot}0945)^2}{175} = 0{\cdot}025068$, leaving as remainder $0{\cdot}028886 - 0{\cdot}025068 = 0{\cdot}003818$.

The two sub-items, linear relation and remainder, of the Times item may now be tested against the interaction mean square, or preferably against the reduced interaction mean square as in Table II*a*. The linear component is clearly signifi-

TABLE II*a*.—*Decomposition of T. and I. × T. Items in Table II.*

Item.	Sum of squares.	Degrees of freedom.	Mean square.	Variance ratio.*	P.
T. Linear relation (L.)	0·025068	1	0·025068	13·97	0·01–0·001
T. Remainder (R.)	0·003818	4	0·000955	—	—
I. × T. Variation in L.	0·024659	4	0·006165	3·44	0·05–0·01
I. × T. Variation in R.	0·028706	16	0·001794	—	—

* Using the variation in R. mean square as the estimate of error.

cant (probability 0·01–0·001), while the remainder mean square is subnormal. There is a significant decline in concentration with time and this can be adequately described using a linear relation on time.

When the mean concentration is calculated for the five individuals separately we can substitute them for $\bar{y}$ in the formula of the straight line to give the relations

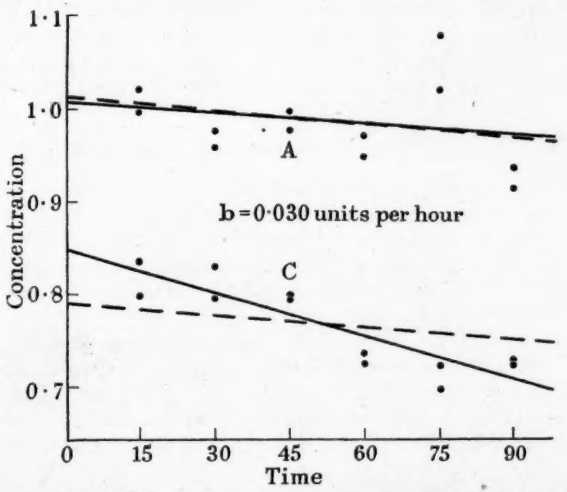


FIG. 1.—Duplicate determinations of dye (in units of mg. dye/100 ml. plasma) at 15, 30, 45, 60, 75 and 90 minutes after injection in two individuals. The solid lines are the best fitting individual straight lines relating concentration to time, and the broken lines that assuming the average range of loss found from 58 individuals. Time is measured in minutes, and b is the rate of loss of dye concentration. Further details in the text.

of concentration and time shown by the separate individuals, assuming all the individuals to have the common slope $b = -0{\cdot}011968$ (Fig. 1). We may, however, inquire whether they in fact agree in the values that they give for b. A value for b, and the sum of squares for which it accounts, can be calculated

from the observation on each individual in just the same way that the common value and sum of squares were found from the totals, except that the factor of 10 is replaced by one of 2 in finding the sum of squares of x. The sum of squares corresponding to the five individual values of b, so obtained, add up to 0·049727 for 5 degrees of freedom, 1 from each of the five individuals. Of this total, 0·025068, for 1 degree of freedom, has already been accounted for by the best fitting common b. This leaves 0·024659 for 4 degrees of freedom for variation between the individual b's. This sum of squares occurs in the original analysis of variance as part of the interaction sum of squares, which may consequently be partitioned. Deducting the sum of squares for variation in b leaves 0·028706 for 16 degrees of freedom. The two mean squares are, therefore, 0·006165 and 0·001794, giving a variance ratio of 3·44 (Table II*a*). For 4 and 16 degrees of freedom, such a variance ratio has a probability of just under 0·05, and so there is good reason to suspect that the decline in concentration with time is not the same in all five individuals. The dye passes out of their plasmas at different speeds. Nor is the rate of loss proportional to the concentration itself, for individuals with higher concentrations do not regularly show higher rates of loss.

In Fig. 1 the observations relating to two of the five men are plotted against time. It will be seen that the duplicate observations show much smaller differences from one another than they do from the lines relating concentration and time. The chemical error is less than the biological. The solid lines are those best fitting the individual data. The broken lines are drawn through the mean concentration, but with the common slope of — 0·030 units (mg. dye/100 ml. serum) per hour, found from the larger body of data to be examined in the next section. The individual rate of loss is lower in A with the higher concentration.

The Standard Error of Concentration Determinations.

After the foregoing analysis was completed, a larger body of individuals was examined in order to determine the natural variation in plasma volume. Since chemical error could be neglected, only single determinations were made at each of two times, 30 and 90 minutes after injection. By using two times an estimate could be obtained of the error variation due to differences between the ratio of loss of dye in different individuals.

Fifty-three male students between the ages of 19 and 25 years were injected in this second test. The previous five were also included in the fuller analysis, their first concentrations at 30 and 90 minutes (Table I) after injection being used for this purpose. Thus a total sample of 58 was obtained. Data for the 53 students are given together with their heights, weights and estimated surface areas in Table III.

The analysis of variance of concentration now falls into only three parts, since the Duplicates item is no longer present. There are 57 degrees of freedom between the 58 individuals, but only 1 degree of freedom between the two sampling times. From the total of 115 degrees of freedom between 116 observations, 57 are thus left for the Individuals-Times interaction. The sums of squares are found in a way analogous to that used for the similar items of the previous test, except that the divisors used are adjusted to the new number of observations summed in obtaining the quantities which are to be required. The total sum of squares is obtained using a divisor of 1 ; that for Individuals using 2 ; that for

TABLE III.—*Data Relating to Plasma Volume Determinations in 53 Male Students Aged 19–25.*

Height (cm.).	Weight (kg.).	Surface area (sq. m.).	Dye injected (mg.).	Concentration at 30 min. (mg. per 100 ml.).	Concentration at 90 min. (mg. per 100 ml.).	Plasma volume (litres).
183	74·6	1·95	36·7	0·80	0·80	4·42
188	79·4	2·04	33·1	0·81	0·77	4·04
168	55·5	1·63	33·1	1·04	1·00	3·15
168	71·2	1·79	33·1	0·85	0·82	3·82
170	60·7	1·69	35·0	1·23	1·25	2·76
169	70·5	1·80	36·5	0·92	1·02	3·65
178	62·5	1·77	33·2	1·02	0·85	3·44
174	60·3	1·71	37·4	0·98	0·88	3·90
178	58·5	1·72	35·0	0·96	1·02	3·43
181	80·7	2·00	37·4	0·96	0·98	3·74
178	71·2	1·86	33·9	0·70	0·70	4·64
179	61·6	1·76	32·9	0·90	0·98	3·39
174	69·4	1·83	36·7	0·92	0·89	3·93
175	61·6	1·74	32·9	0·94	0·96	3·36
174	74·8	1·89	32·9	0·92	0·94	3·43
174	76·2	1·90	33·9	0·83	0·95	3·68
177	68·0	1·83	33·2	0·86	0·75	3·98
185	73·5	1·95	33·9	0·78	0·73	4·32
163	53·5	1·56	33·9	1·02	0·80	3·61
170	61·2	1·69	33·2	0·87	0·78	3·88
173	72·6	1·85	35·9	1·00	1·02	3·45
180	68·4	1·85	32·9	0·88	0·82	3·74
179	76·2	1·93	35·7	0·74	0·78	4·52
166	64·4	1·70	32·9	0·98	0·86	3·46
178	77·6	1·94	33·1	0·99	0·84	3·50
170	60·3	1·68	35·9	0·94	0·82	3·95
170	60·3	1·68	35·7	1·20	1·16	2·95
176	78·5	1·94	35·9	0·83	0·86	4·10
182	73·9	1·94	33·1	0·84	0·83	3·83
177	69·9	1·85	35·7	0·94	1·02	3·53
188	87·1	2·12	32·9	0·72	0·74	4·33
179	73·0	1·91	33·2	0·82	0·80	3·95
174	71·7	1·85	33·2	0·94	0·87	3·55
185	81·2	2·04	37·4	0·88	0·78	4·35
164	66·7	1·72	33·2	0·99	0·96	3·30
178	72·6	1·89	35·7	0·86	0·72	4·35
177	65·8	1·81	37·4	1·00	1·08	3·50
177	61·0	1·73	33·2	1·02	1·00	3·19
193	76·2	2·04	32·9	0·51	0·53	5·98
156	54·4	1·51	36·5	1·21	1·31	2·83
187	72·1	1·94	36·5	0·88	0·98	3·80
180	69·6	1·88	32·9	0·73	0·70	4·42
166	63·9	1·70	35·0	1·33	1·36	2·55
183	75·2	1·96	33·9	1·02	0·98	3·29
177	64·4	1·78	36·7	0·94	1·08	3·53
183	74·4	1·94	36·5	0·94	0·94	3·76
182	71·7	1·91	35·9	1·25	0·82	3·37
171	57·2	1·67	35·0	1·08	1·00	3·27
174	71·7	1·85	36·7	0·92	0·90	3·90
179	69·4	1·86	33·1	0·66	0·70	4·66
168	60·8	1·68	33·9	0·94	0·94	3·49
177	65·3	1·79	33·2	0·97	0·68	3·88
161	49·9	1·50	35·9	1·21	1·25	2·85

Times using 58; and the correction term using 116. The analysis showing the mean squares is given in Table IV.

The mean square for Times is 0·025801 as compared with 0·025068 for the corresponding linear item obtained previously, and that for the interaction is

TABLE IV.—*Analysis of Variance of Dye Concentrations in 58 Individuals.*

Item.	Sum of squares.	Degrees of freedom.	Mean square.	Variance ratio.	P.
Individuals	2·526828	57	0·044330	8·91*	V. small
Times	0·025801	1	0·025801	5·19*	0·05–0·01
Interaction	0·283649	57	0·004976	2·77†	0·01
Total	2·836278	115			

* Using the interaction mean square as the estimate of error.
† Using the " Variation in R " mean square of Table II*a* as the estimate of error (see text).

0·004976 as compared with 0·006165 (variation in L in Table II*a*). In neither case is the difference significant. The Times item is significantly larger than the interaction, with a probability of about 0·02. The interaction item gives a variance ratio of 2·77 with the reduced interaction mean square of 0·001794 from the earlier analysis. This variance ratio has a probability of just over 0·01, and so must be judged significant. Since the present interaction item corresponds with that portion of the former interaction testing the variation in rate of loss of dye between individuals, the new analysis confirms, on a wider range of material, that individuals lose dye at different rates. The average rate of loss is, however, smaller than before. The total loss between 30 minutes and 90 minutes over all 58 subjects is 1·73 units (i.e. mg. dye/100 ml. serum), or 0·029828 units per hour per individual. Since the mean sampling time is 60 minutes, 0·030 units must therefore be added to the mean of the two samples from one individual in order to estimate the concentration at zero time.

The variance of a single observed concentration (V_y) estimated by the interaction mean square is 0·004976. The variance of the mean of the two observations, made on one individual at 30 and 90 minutes, is thus $1/2 \times 0{\cdot}004976$ or 0·002488 (Mather, 1946, Section 32). The variance of b, the rate of loss which we have now measured in time units of 1 hour, is $\frac{V_y}{S(x-\bar{x})^2}$. The sample times are each 1/2 hour from their mean and so each individual contributes $1/4 + 1/4$ to the sum of squares of x. Since 58 subjects were used, $S(x-\bar{x})^2 = 58 \times 1/2$ or 29, so giving $V_b = \frac{0{\cdot}004976}{29} = 0{\cdot}0001716$.

Now the concentration given by the linear relation for any time x is found as $Y = \bar{y} + b(x-\bar{x})$, where $\bar{y}$ is the mean of the observed concentration. V_y is therefore $V_{\bar{y}} + V_b\,(x-\bar{x})^2$. $V_{\bar{y}}$ has been found as 0·002488 and V_b as 0·000172. Since zero time ($x = 0$) is 1 hour from $\bar{x}$, the mean of the sample time, so giving $(x-\bar{x})^2 = 1$, the variance of the estimated concentration at zero time is $V_{Yo} = V_{\bar{y}} + V_b = 0{\cdot}002660$. The standard error ($s_{Yo}$) of the estimated concentration at zero time is found as $s_{Yo} = \sqrt{V_{Yo}} = 0{\cdot}051573$ or 0·0516 approximately.

The initial concentration of an individual is thus estimated by adding 0·030 to the mean of the two observations and is subject to a standard error of 0·0516. It should be noted that this applies only to cases where concentrations are determined at 30 and 90 minutes after injection, though corresponding corrections and standard errors can be calculated from the data given by methods analogous with those used, for cases where other numbers of observations are made and at other times.

Validity of the Estimation of Initial Concentrations.

The validity of the estimates of initial concentration, arrived at by the above method, has been called into question by the work of Cruikshank and Whitfield (1944). They describe an experiment in which the relation of dye concentration to time was followed in the cat. Their results suggested that dye was rapidly lost from the plasma, presumably to the reticulo-endothelial sytem, in the first 5–6 minutes after injection. The fall was then of the familiar type, but estimates of original concentration made from later concentrations in the plasma would obviously be too low. The system which was responsible for this early loss became saturated, however, so that a true estimate of the change of concentration in the plasma could be made following a second injection.

The occurrence of such an effect in man was tested by the injection of 6 individuals with dye. The concentration was determined in samples of plasma taken at 20, 40 and 60 minutes after injection. A second injection, equal to the first, was made 60 minutes after the last of the three samples was taken, i.e. 120 minutes after the first injection. Three further samples were taken for determinations of dye concentrations at 20 minute intervals thereafter, i.e. at 140, 160 and 180 minutes after the experiment started. The results are given in Table V.

TABLE V.—*Double Injection Test. Second Injection Equal to and 2 Hours after First, from which All Times are Measured.*

Time in minutes.	Sample.	Individual.						Total.	Omitting 3.
		1.	2.	3.	4.	5.	6.		
20	A_1	0·77	0·72	1·20	1·07	1·13	0·96	5·85	4·65
40	A_2	0·80	0·70	1·00	1·02	1·06	0·92	5·50	4·50
60	A_3	0·73	0·70	1·22	0·95	0·96	0·94	5·50	4·28
140	A_7	1·44	1·33	2·94	2·01	2·01	1·78	11·51	8·57
160	A_8	1·43	1·32	2·91	2·05	2·05	1·80	11·56	8·65
180	A_9	1·40	1·33	2·87	1·97	1·84	1·81	11·22	8·35
Sum of squares for cat effect		0·0002	0·0078	0·0808	0·0036	0·0075	0·0015	0·0206	0·0008

Without entering into the details of the analysis of variance of these data, it can be said that in all individuals the rates of loss of dye were not detectably different following the second injection as compared with the first. The interaction mean square, appropriate for use as an estimate of error variation, was found to be 0·0031 for 20 degrees of freedom as compared with 0·0050 for 57 degrees of freedom in the previous experiment. This difference is insignificant.

Measuring time in units of 20 minutes, we have concentrations A_1, A_2 and A_3 found at times 1, 2 and 3 after the first injection. The second injection was then made at time 6, and concentrations A_7, A_8 and A_9 subsequently found at times 7, 8 and 9. Since b, the rate of loss of dye per unit time, may be taken as unaffected by the second injection, it is best estimated as $b = 1/2[\frac{1}{2}(A_1 - A_3) + 1/2(A_7 - A_9)] = 1/4(A_1 - A_3 + A_7 - A_9)$. The concentration expected at time 2 will be the mean of A_1, A_2 and A_3, and that at time 8 will be the mean of A_7, A_8 and A_9. If there is no effect of the kind found in the cat, i.e. that the consequences of the two injections are simply additive, the concentration at time 2 will be less than that at time 0, when the first injection was made, by an amount

equal to that by which concentration at time 8 is less than that at time 6, when the second injection was made. Now by time 6, the concentration produced by the first injection, which may be denoted by c, will be reduced by $6b$ to $c - 6b$. The second injection then raises it by c to $2c - 6b$. Thus c may be estimated in two ways: First, since the mean of A_1, A_2 and A_3 is $c - 2b$, $c = 1/3(A_1 + A_2 + A_3) + 1/2(A_1 - A_3 + A_7 - A_9)$. Second, since the mean of A_7, A_8 and A_9 is $2c - 6b - 2b$, $2c = 1/3(A_7 + A_8 + A_9) + 2(A_1 - A_3 + A_7 - A_9)$. The agreement or otherwise of these two estimates is the necessary test of the blocking effect found in the cat. With no blocking effect they will be equal within the limits of error variation. With such a blocking effect the former estimate will be lower than the latter.

If the two estimates of c are in agreement—

$$1/3\ (A_1 + A_2 + A_3) + 1/2\,(A_1 - A_3 + A_7 - A_9) = 1/6\,(A_7 + A_8 + A_9) + (A_1 - A_3 + A_7 - A_9),$$

or $A_1 - 2A_2 - 5A_3 + 4A_7 + A_8 - 2A_9 = 0$.

The sum of squares of the coefficients of $A_1 \ldots A_9$ in this expression is 51. We may therefore calculate $1/51\ (A_1 - 2A_2 - 5A_3 + 4A_7 + A_8 - 2A_9)^2$ from each individual as a sum of squares for 1 degree of freedom. If there is no blocking effect this sum of squares will not exceed the value permissible by comparison with the error variance when measured by the appropriate interaction mean square. A significantly great blocking effect will be indicated by a significant excess of this squared comparison over the error variance.

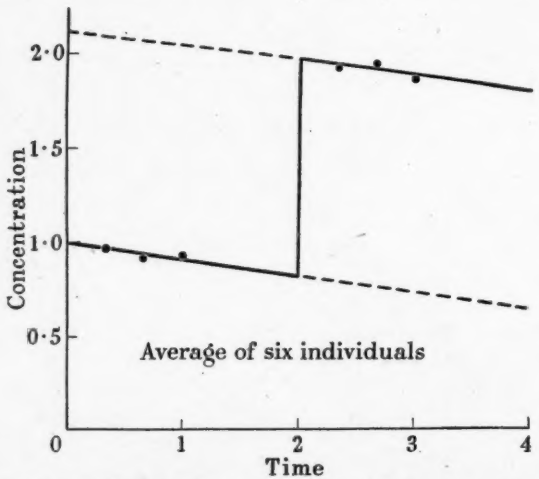


FIG. 2.—A test on six individuals of the occurrence of anomalous loss of dye after the first injection, as found in the cat. The points are the averages of determinations on six individuals, concentration being measured in mg. dye/100 ml. plasma and time in units of 20 minutes. The second injection suggests a lower plasma volume than the first. This is due to the behaviour of an exceptional individual (see text).

The sum of squares given by each of the six individuals from this comparison is shown in Table V. They total 0·1014 for 6 degrees of freedom. Performing the same calculation on the totals for the six individuals, and introducing a

factor of 6 into the divisor of the comparison to adjust for the use of such totals, gives a sum of squares of 0·0206 for 1 degree of freedom. This tests the overall tendency to show the effect found in the cat. Subtracting it from the pooled sum of squares leaves 0·0808 for 5 degrees of freedom, which tests the uniformity of the 6 individuals in showing, or failing to show, the effect. The error mean square from the appropriate interaction items was 0·0031, which gives variance ratios of 6·64 and 5·22 with the mean squares testing the total cat effect and the uniformity. The former has a probability of between 0·05 and 0·01, and the latter a probability of less than 0·01. Taking the larger error mean square of 0·0050 from the experiment on 58 individuals, the two variance ratios are 4·12 and 3·24, both of which have probabilities between 0·05 and 0·01. There is clearly a suggestion of the cat effect, but the 6 individuals are not in agreement.

Inspection of Table V suggests the reason for this state of affairs. Individual 3 appears to differ in showing the effect from the rest, who do not. His contribution to the pooled sum of squares is 0·0808 out of 0·1014. If he is omitted the remainder give a total of 0·0206 for 5 degrees of freedom, which is partitioned into a general deviation item of 0·0008 for 1 degree of freedom and an item of 0·0198 for 4 degrees of freedom, testing uniformity. Neither is significant. These five show no effect of the kind found in the cat, but individual 3 clearly does so.

The position is thus: that usually plasma volumes determined from single injections will be valid; but that the effect found in the cat must be expected occasionally, with a consequent under-estimate of concentration and over-estimate of plasma volume.

Natural Variation in Plasma Volume.

Plasma volumes have been calculated from the data on the 53 students (Table III). The first one, for example, received an injection of 36·7 mg. of dye. Both concentration determinations gave values of 0·80 mg. dye per 100 ml. serum, so that the concentration at zero time is estimated as $1/2\ (0{\cdot}80 + 0{\cdot}80) + 0{\cdot}030$ or 0·830 mg./100 ml. The plasma volume is then clearly to be found as $\frac{36{\cdot}7}{0{\cdot}830 \times 10}$ or 4·42 litres. The remainder are calculated similarly.

The lowest plasma volume recorded was 2·55 litres and the highest 5·98 litres. Fig. 3 shows the frequency distribution of the 53 observations grouped in units of 0·2 litres centred on 2·6, 2·8, 3·0 . . . 6·0 litres. The mean value of 3·73 litres is marked by an arrow. Apart from the single observation of 5.98 litres, the distribution is no more irregular than would be expected from a sample of this size; but there is an interval of 1·34 litres between this topmost observation and the next highest at 4·64. The records of height, weight and surface area suggest nothing to explain this remarkably high value of 5·98, the validity of which must therefore fall under suspicion. A possibility which immediately suggests itself is that the individual in question was showing the blocking effect found in the cat. As we have seen, this effect would lead to a serious over-estimate of plasma volume. Taking the exceptional individual of Table V as the basis for estimating the strength of the effect, it appears that the concentration at zero time might be well estimated at little more than half its correct figure, so that the plasma volume might appear as anything up to nearly twice its true value. Such an overestimate would be of the magnitude indicated by the dis-

crepancy between 5·98 litres and the mean of 3·73 litres. The explanation is therefore reasonable. It is reassuring that only one of fifty-three students suggests the occurrence of this effect leading to over-estimation. The cause of this exceptional behaviour is not apparent, though it may possibly be genetical.

The sum of squares of deviations of the 53 plasma volumes from their mean is 17·2087, for 52 degrees of freedom. This value is, of course, affected by the inclusion of the individual apparently having 5·98 litres; but in the absence of certainty that this value is spuriously large by reason of under-estimation of

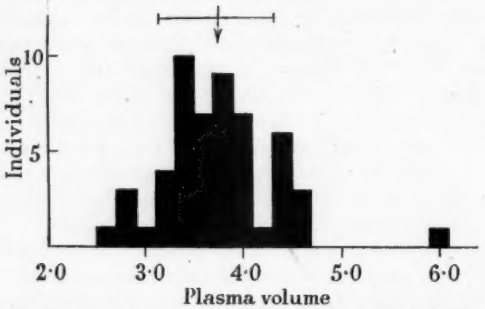


FIG. 3.—Frequency distribution of plasma volumes, in litres, of 53 students, grouped in classes of 0·2 litres centred on 2·6, 2·8, 3·0, etc., litres. The arrow marks the mean and the bar on each side of it the standard deviation of the distribution. The single exceptionally high value may be due to under-estimation of concentration resulting from anomalous loss as observed in the cat.

dye concentration at zero time, it seemed advisable not to reject it and to accept a possibly inflated estimate of the variance. Dividing the sum of squares by the degree of freedom gives a mean square of 0·331 as the estimated variance of plasma volume. The standard deviation, found as the square root of the mean square, and marked in Fig. 3 by a bar on each side of the mean, is therefore 0·575. The mean itself will have a variance of $\frac{0{\cdot}331}{53}$ or 0·00624 and a standard error of $\sqrt{0{\cdot}00624}$ or 0·079.

A further value is of interest, viz. the standard error of the determination of the plasma volume of a single individual. The standard error of the estimated dye concentration at zero time has been found as 0·05157. Now the general rule relating the standard errors of two quantities derivable from each other is—

$$s_y^2 = s_x^2 \left(\frac{dy}{dx}\right)^2.$$

Where v is plasma volume, c is estimated dye concentration and d is dye injected we find—

$$v = \frac{d}{10c} \text{ so that } \frac{dv}{dc} = -\frac{d}{10c^2}.$$

Then—

$$s_v^2 = s_c^2 \frac{d^2}{100c^4} \text{ and } s_v = s_c \frac{d}{10c^2}.$$

Since d and c varied from individual to individual, the plasma volume must be found with varying precision, even though s_c may be taken as constant. For the first individual of Table III $d = 36{\cdot}7$ and $c = 0{\cdot}830$, so that $s_v = 0{\cdot}05157 \frac{36{\cdot}7}{10 \times 0{\cdot}830^2} = 0{\cdot}2737$. Combining the 53 standard errors, by averaging the variances of which they are the square roots, gives the mean standard error as 0·2247.

Plasma volume might be expected to be related to dimensions of the body, though whether the relation would be strongest with linear, quadratic or cubic measures of body size is not easily foreseeable. Height in cm. and weight in kg. were determined for each of the 53 students at the time plasma volume was estimated. Surface area in square metres was then found from Boothby and Sandford's nomogram.

These data are given with plasma volume in Table III, from which the corresponding correlations were found as follows :

Correlations of plasma volume with—

Height	0·64
Area	0·63
Weight	0·55

All are highly significant, and there is little to choose between them, though, for what it is worth, the correlation decreases with the power of the dimensions used. None of the correlations is of any great value for predicting plasma volume, other than pointing to the obvious expectation that the larger the individual the greater the plasma volume. The best of them, that with height, only accounts for a sum of squares of 7·07 out of the 17·21 for plasma volume between individuals. In other words, 59 per cent of the variation between individuals in plasma volume cannot be ascribed to corresponding differences in height. Combining height and weight to give the best joint predictive still leaves 57 per cent of the variation in plasma volume unaccounted for. Area is determined from a nomogram relating it to height and weight, and so will add little to the accuracy of any predictive of plasma volume. We are, therefore, led to conclude that bodily dimensions cannot profitably be used as a means of predicting plasma volume.

SUMMARY.

Five individuals were injected with Evans blue and the concentration of dye in their plasmas determined by duplicate observations at 15-minute intervals up to 90 minutes after injection. Dye is slowly lost from the plasma system, the loss being linearly related to time. The rate of loss varies between individuals. The technical error as measured by differences between duplicate observation was smaller than the biological variation, as measured by departure from the concentration expected on the linear relation.

The variation in rate of loss was confirmed on 53 male students whose dye concentrations were determined 30 and 90 minutes after injection. The average rate of loss was 0·030 mg. dye per 100 ml. serum per hour. With determination of concentration at 30 and 90 minutes after injection, the concentration at zero time is best estimated by the mean of the determination plus 0·30, and has a

standard error of 0·052. The method of calculating the correction for dye loss with time and the standard error of zero time estimate can be applied to other systems of dye determination times.

A test is described on six men of the existence of the effect, discovered in the cat, which leads to under-estimation of zero time concentration. Five of the six showed no sign of the effect, but the sixth gave evidence of its occurrence. Determinations of zero time concentrations by the customary method will therefore be valid generally, though subject to under-estimation occasionally.

The plasma volumes of the 53 students had a mean of 3·73 ± 0·079 litres, with a standard deviation of 0·575 litres. The lowest observed was 2·55 litres, and the highest, with one exception, 4·64 litres. The exception was 5·98 litres—a value which may be inflated by the occurrence of the cat effect. The standard error of a plasma volume determination varies with the volume itself and with the amount of dye injected, but averaged 0·2247 in the cases observed.

Plasma volume shows a correlation of 0·64 with height, 0·63 with surface area and 0·55 with weight in the 53 students. None of these measurements is a useful predictive of plasma volume.

REFERENCES.

CRUIKSHANK, E. W. H., AND WHITFIELD, I. C.—(1944) *Proc. physiol. Soc.*, **103**, 198.
MATHER, K.—(1946) 'Statistical Analysis in Biology.' London (Methuen, 2nd ed.).
MORRIS, C. J. O. R.—(1944) *Biochem. J.*, **38**, 203.

ANTICOLLAGENASE IN IMMUNITY TO *CL. WELCHII* TYPE A INFECTION.

D. G. EVANS.

From the National Institute for Medical Research, Hampstead.

Received for publication November 22, 1946.

THE relative importance of α toxin, θ haemolysin and hyaluronidase in experimental *Cl. welchii* A infection, and of their respective antibodies in controlling the disease, has been the subject of a series of investigations during recent years (Evans, 1943*a* and *b*, 1945*a* and *b*). It was found that antisera containing α antitoxin were highly effective in protecting guinea-pigs against infection with a number of virulent strains of *Cl. welchii* A, whether the sera contained θ antihaemolysin and antihyaluronidase or not. On the other hand, antisera containing either θ antihaemolysin or antihyaluronidase but only a trace of α antitoxin had no influence on the course of the infection. Nor did they enhance the protective action of α antitoxin. It was also shown with 30 strains of *Cl. welchii* A from a variety of sources that the ability of a strain to produce fatal infection in guinea-pigs was related to the production of α toxin but not to the production either of θ haemolysin or hyaluronidase. These

results suggested that α toxin played the most important rôle in *Cl. welchii* A infection, and that α antitoxin was the significant antibody in the control of the disease.

Recently attention has been drawn to another antigenic enzyme produced by *Cl. welchii* A. Macfarlane and MacLennan (1945) observed that filtrates from broth cultures of *Cl. welchii* A were able to break down muscle by their action on collagen. They regarded the active substance as a collagenase, and suggested that it was responsible for the muscle destruction observed in gas gangrene. They further suggested that an antitoxin or toxoid designed to confer a high anticollagenase immunity might be more effective in the control of gas gangrene produced by *Cl. welchii* A than the present immunizing agents, which are judged by their α antitoxin effect. More recently Oakley, Warrack and van Heyningen (1946) found by *in vitro* tests that the collagenase was immunologically distinct from the other known antigens present in *Cl. welchii* A filtrates, and designed methods for determining the anticollagenase values of *Cl. welchii* antisera.

In view of this recent work, further tests have been made to investigate the protective action of anticollagenase in experimental gas gangrene. In the previous studies no account was taken of the anticollagenase activity of the antisera which were then employed, since the possible importance of this antibody was not appreciated at the time.

METHODS.

Two natural, unconcentrated *Cl. welchii* antisera prepared in horses at the Wellcome Laboratory have been employed. (1) R 6423 contained 75 units of α antitoxin per c.c. but no detectable anticollagenase, and (2) R 5434 contained 50 units of anticollagenase and only 0·2 units of α antitoxin per c.c. The arbitrary unit of anticollagenase is that chosen by Oakley and his colleagues. In the majority of the experiments antiserum was administered intramuscularly to guinea-pigs 20 hours *before* the infecting dose of organisms was given ; in one experiment the antiserum was given intravenously 4 hours *after* infection. Guinea-pigs having an average weight of 450 g. were used, and kept under observation for at least 7 days after the onset of infection. Four virulent strains of *Cl. welchii* A were used, S.R. 9, S. 107, S.R. 12 and A. 118, all producing collagenase *in vitro*.

Three different methods of producing experimental gas gangrene were employed. In each a suspension of washed bacilli was prepared from a 3-hour liver broth culture which was centrifuged and the deposit of organisms washed once in saline, centrifuged, and resuspended in saline to give a concentration of about 250×10^6 organisms per c.c. From this suspension 10-fold dilutions in saline were made for injection.

In method 1 a dose of 0·2 c.c. of washed organisms was injected intramuscularly into the thigh of the animal 3 hours after an injection of $CaCl_2$ solution into the same site (Evans, 1943*a*).

Method 2 was based on the observation of Cooper (1946) that the infectivity of *Cl. welchii* was increased considerably when the organisms were injected with adrenaline ; 0·2 c.c. of the bacterial suspension was injected about 10 minutes after an intramuscular injection of 0·5 c.c. of a 1 : 10,000 dilution of adrenaline

into the same site. This method produced fatal gas gangrene with dilute bacterial suspensions, and the course of the disease was similar to that observed when $CaCl_2$ was used as the adjuvant.

Method 3 was designed to produce a local skin infection. The bacterial suspension was mixed with equal parts of a 1 : 50,000 dilution of adrenaline and 0·2 c.c. of the mixture injected into the depilated skin of guinea-pigs. With small numbers of organisms a necrotic, oedematous infected lesion developed, which was often arrested after 24 hours, whereas with larger numbers the infection sometimes spread extensively and occasionally caused the death of the animal. With this method the protective effect of antisera was estimated on the smallest dose of organisms able to produce a skin infection.

RESULTS.

Experiments Using Calcium Chloride as Adjuvant.

Protective properties of anticollagenase.—The protective action of anticollagenase (R 5434) was tested as in Table I. Guinea-pigs were infected with *Cl. welchii* (S.R. 9) 20 hours after the intramuscular injection of serum. No protection was afforded by 50 units of anticollagenase. This result is confirmed by

TABLE I.—*Resistance of Guinea-pigs to Infection Produced by* Cl. welchii *A* (*S.R.* 9) *and* $CaCl_2$ *after Receiving* (a) *No Serum, and* (b) *Anticollagenase.*

Dilution of *Cl. welchii* Suspension (0·2 c.c.).	Result with guinea-pigs (2 in each group) receiving—	
	No serum.	50 units of anticollagenase.
10^{-5} .	D 4 ; D 1	D 1 ; D 2
10^{-6} .	D 1 ; S +++	D 4 ; D 3
10^{-7} .	D 2 ; D 5	D 4 ; S +++
10^{-8} .	S − ; S −	S ++ ; S −

Key to Tables I–VI :
D 2 = death in 2 days ;
S = survival ;
− = no reaction ;
+ = small local swelling ;
++ = large gangrenous lesion ;
+++ = extensive gangrene.

an observation from the previous investigations (Evans, 1943*a* and *b*) with an antiserum (R 5432) containing only a trace of α antitoxin, which has since been shown by Dr. Oakley to contain anticollagenase. The largest dose given (2 c.c.) now proves to have contained 80 units of anticollagenase, and was quite ineffective in protecting animals against infection by a number of strains of *Cl. welchii* A.

Protective properties of α antitoxin.—In a similar manner the protective properties of α antitoxic serum containing no anticollagenase (R 6423) was investigated with four strains of *Cl. welchii* A. Guinea-pigs receiving 50 units of α antitoxin were protected against at least 10,000 average lethal doses of *Cl. welchii* (S.R. 9) (Table II). One infecting dose only of strains S. 107, A. 118

TABLE II.—*Resistance of Guinea-pigs to Infection Produced by* Cl. welchii *A* (*S.R.* 9) *and* $CaCl_2$, *after Receiving* (a) *No Serum, and* (b) α *Antitoxin.*

Dilution of *Cl. welchii* suspension (0·2 c.c.).	Result with guinea-pigs (2 in each group) receiving—	
	No serum.	50 units of α antitoxin.
10^{-3}	..	S +; S —
10^{-4}	D 3; D 2	S —; S —
10^{-5}	D 1; D 2	S —; S —
10^{-6}	D 4; D 2	S —; S —
10^{-7}	D 2; D 2	S —; S —

TABLE III.—*Resistance of Guinea-pigs to Infection Produced by* Cl. welchii *A* (3 *Strains*) *and* $CaCl_2$, *after Receiving,* (a) *No Serum, and* (b) α *Antitoxin.*

Strain.	Dilution of *Cl. welchii* Suspension (0·2 c.c.).	Result with guinea-pigs (3 in each group) receiving	
		No serum.	50 units of α antitoxin.
S.R. 12	10^{-3}	D 3; D 5; D 4	S —; S —; S —
S. 107	10^{-1}	D 3; D 4; D 4	S —; S —; S —
A. 118	10^{-3}	D 3; D 2; D 3	S —; S —; S —

and S.R. 12 was used, containing approximately 1000 average lethal doses, and again a dose of serum containing 50 units of α antitoxin and no anticollagenase protected the animals against fatal infection (Table III).

Protective properties of α antitoxin and anticollagenase combined.—An experiment was designed to observe whether anticollagenase would enhance the protective action of α antitoxin (Table IV). Guinea-pigs in one group were given graded doses of α antitoxin, and those in a second group the same doses of α antitoxin to which had been added 50 units of anticollagenase. Twenty

TABLE IV.—*Resistance of Guinea-pigs to Infection Produced by* Cl. welchii *A* (*S.R.* 9) *and* $CaCl_2$ *after Receiving* (a) α *Antitoxin, and* (b) α *Antitoxin and Anticollagenase.*

Dose of α antitoxin (units).	Result with guinea-pigs (2 per dose) receiving approximately 100 lethal doses *Cl. welchii.*	Dose of α antitoxin and anticollagenase (K) (units).	Result with guinea-pigs (2 per dose) receiving approximately 100 lethal doses *Cl. welchii.*
40 α	S —; S —	40 α + 50 K	S —; S —
20 α	S —; S +	20 α + 50 K	S +; S +
10 α	S +; D 3	10 α + 50 K	S +; D 4
5 α	S +++; D 1	5 α + 50 K	D 4; D 2
2·5 α	D 1; D 4	2·5 α + 50 K	S +++; D 4
1·25 α	D 4; D 2	1·25 α + 50 K	D 2; D 4

hours later each guinea-pig was injected with 100 average lethal doses of *Cl. welchii* (S.R. 9) as determined by a simultaneous test on unprotected animals. Antiserum containing from 10 to 20 units of α antitoxin but no anticollagenase was protective, but no more so when given together with 50 units of anticollagenase.

Curative properties of α antitoxin.—A test was made, similar to those described previously (Evans, 1945*b*), of α antitoxin given intravenously to guinea-pigs *after* they had been infected with *Cl. welchii* A (Table V). Each of 18 guinea-

TABLE V.—*Treatment of Guinea-pigs with α Antitoxic Serum Containing No Anticollagenase 4 Hours after Infection with* Cl. welchii *A (S.R. 9) and* $CaCl_2$.

Dose of *Cl. welchii*.	Dose of intravenous α antitoxin (units).	Result (3 guinea-pigs per dose).
Approximately 100 lethal doses	2·5	D 4; D 3; D 5
	5	D 5; D 2; D 2
	10	S +++; S ++; S ++
	20	S +++; S ++; D 6
	40	S ++ ; S ++; S ++
	80	D 3 ; S + ; S +

pigs was infected with approximately 100 average lethal doses of S.R. 9, as determined by a simultaneous test on untreated animals. Four hours later varying doses of α antitoxin (R 6423) were given intravenously. It was evident that the α antitoxic serum containing no anticollagenase gave substantial protection against fatal gas gangrene when given after the onset of infection.

Experiments Using Adrenaline as Adjuvant.

Intramuscular infection.—A comparison was made of the protective action of anticollagenase and α antitoxin in fatal infection produced by *Cl. welchii* A activated by adrenaline (Table VI). Twelve animals each received 50 units

TABLE VI.—*Resistance of Guinea-pigs to Infection Produced by* Cl. welchii *A (S.R. 9) and Adrenaline after Receiving* (a) *No Serum,* (b) α *Antitoxin, and* (c) *Anticollagenase.*

Dilution of *Cl. welchii* suspension (0·2 c.c.).	Result with guinea-pigs (2 in each group) receiving—		
	No serum.	50 units of α antitoxin.	50 units of anticollagenase.
10^{-2}	D 2 ; D 2	S ++; S +	D 1 ; D 2
10^{-3}	D 2 ; D 2	S +; S +	D 4 ; D 1
10^{-4}	D 2 ; D 3	S +; S −	D 1 ; D 2
10^{-5}	D 4 ; D 3	S −; S −	S ++; D 2
10^{-6}	S +; D 2	S −; S −	D 2 ; D 2
10^{-7}	S −; S −	S −; S −	S +; S −

of α antitoxin (R 6423), 12 each received 50 units of anticollagenase (R 5434), and 12 were not given serum. Twenty hours later all 36 animals were each injected intramuscularly with 0·5 c.c. of a 1 : 10,000 dilution of adrenaline, and about 10 minutes later washed *Cl. welchii* organisms (S.R. 9) were injected in the same site. A dose of 50 units of α antitoxin protected against approximately 10,000 average lethal doses (the highest infecting dose used), but 50 units of anticollagenase gave no protection whatsoever against the smallest lethal dose.

Intradermal infection.—A similar result was obtained with intradermal infection by mixtures of *Cl. welchii* A and adrenaline. Groups of guinea-pigs were immunized as shown in Table VII. Twenty hours later each animal was

TABLE VII.—*Resistance of Guinea-pigs to Skin Infection Produced by* Cl. welchii *A (S.R. 9) and Adrenaline after Receiving* (a) *No Serum,* (b) α *Antitoxin, and* (c) *Anticollagenase.*

Dose of serum to each guinea-pig.	Skin lesions at 24 hours after receiving following dilutions of *Cl. welchii* suspension.						Number of guinea-pigs.	Number of deaths.
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}		
70 units α antitoxin	+	−	−	−	−	−	3	0
30 „ „	+	−	−	−	−	−	3	0
10 „ „	+	−	−	−	−	−	3	0
100 „ anticollagenase	+++	+++	+++	+++	+++	−	3	1
50 „ „	+++	+++	+++	+++	+++	−	3	2
None	+++	+++	+++	+++	+++	−	3	2

Key to Table VII :

− = no reaction ;
+ = inflammatory reaction ;
+++ = progressive infected lesion with necrosis.

given six intradermal injections of 10-fold dilutions (10^{-1} to 10^{-6}) of a washed *Cl. welchii* (S.R. 9) suspension (250×10^6 organisms per c.c.) mixed with equal parts of a 1 : 50,000 dilution of adrenaline. The minimal infecting dose was a dilution of 10^{-5}. All the animals receiving α antitoxin were protected against 1000 infecting doses of *Cl. welchii*, and even the injection of 10,000 infecting doses resulted only in a small indurated area which did not spread. None of the guinea-pigs receiving either 50 or 100 units of anticollagenase was protected against the smallest skin infecting dose (10^{-5} dilution), and all developed extensive infected lesions similar to those shown by the controls. Three of the animals receiving anticollagenase and two in the control group died in 5 to 6 days, but there were no deaths in the groups receiving α antitoxin.

CONCLUSIONS.

A number of antigens have been identified in *Cl. welchii* A filtrates : α toxin, θ haemolysin, hyaluronidase and collagenase. The relative importance of these antigens in *Cl. welchii* A gas gangrene, and of their respective antibodies in the control of the disease, is indicated by the fact that small doses of α antitoxin are effective in protecting guinea-pigs against fatal infection, whereas large doses of antisera containing either θ antihaemolysin, antihyaluronidase or anticollagenase, but no α antitoxin, are quite ineffective. Moreover, neither θ anti-

haemolysin, antihyaluronidase nor anticollagenase enhance the protective properties of α antitoxin.

It therefore appears that in the control of experimental gas gangrene in guinea-pigs α antitoxin is of prime importance. It is also reasonable to suppose that α toxin plays the most important part in the infectious process, and while it is recognized that the rapid spread of the disease may be associated with hyaluronidase production and that muscle destruction may be a result of the action of collagenase, there is so far no evidence to support the view that either of these enzymes plays any substantial part in the genesis of fatal gas gangrene.

SUMMARY.

Fatal gas gangrene was produced in guinea-pigs by injecting washed cultures of *Cl. welchii* A intramuscularly after an injection into the same site of (*a*) $CaCl_2$ solution or (*b*) adrenaline solution. A local skin infection was produced in guinea-pigs by the intradermal injection of a mixture of a washed culture of *Cl. welchii* A suspended in a dilute solution of adrenaline.

The protective action of α antitoxin and anticollagenase occurring in *Cl. welchii* A antisera was tested in these three types of infection. Antiserum containing α antitoxin and no anticollagenase was highly effective in protecting guinea-pigs against infection, whereas an antiserum containing anticollagenase but no α antitoxin was able neither to protect against infection nor enhance the protective properties of α antitoxin.

I am indebted to Dr. C. L. Oakley for the sera used in this investigation.

REFERENCES.

Cooper, E. V.—(1946) *Lancet*, i, 459.

Evans, D. G.—(1943*a*) *Brit. J. exp. Path.*, **24**, 81.—(1943*b*) *J. Path. Bact.*, **55**, 427.—(1945*a*) *Ibid.*, **57**, 75.—(1945*b*) *Brit. J. exp. Path.*, **26**, 104.

Macfarlane, R. G., and MacLennan, J. D.—(1945) *Lancet*, ii, 328.

Oakley, C. L., Warrack, G. H., and van Heyningen, W. E.—(1946) *J. Path. Bact.*, **58**, 229.

AN ANTIBIOTIC FROM *PENICILLIUM TARDUM*.

N. BORODIN, F. J. PHILPOT* AND H. W. FLOREY.

From the Sir William Dunn School of Pathology, Oxford.

Received for publication November 29, 1946.

WILKINS and Harris (1943) reported that culture filtrates from *Penicillium tardum* (National Collection of Type Cultures No. 1719) showed antibiotic activity. The purification of an antibiotic substance produced by this mould and some of its chemical and biological properties are described. For convenience it will be referred to as tardin.

EXPERIMENTAL.

Production and Purification.

The mould was grown on 2 per cent malt extract in tap water, at 24° C., in vessels which each contained about 1·2 litres of culture medium in a layer 2 cm. deep. The antibacterial activity was tested by the cylinder-plate method (Abraham, Chain, Fletcher, Florey, Gardner, Heatley and Jennings, 1941), using plates seeded with a 24-hour culture of *Staph. aureus* (N.C.T.C. No. 6571) diluted 1 in 1000.

A maximum zone of inhibition, 20 mm. in diameter, was reached in 9 days when the medium was pH 4. The vessels were harvested between the 9th and 12th days. The activity remained constant up to 15 days.

The medium, after being freed from mycelium by filtration, was adjusted to pH 7 and shaken with an equal volume of amyl acetate. After separation in a Sharples centrifuge the amyl acetate was distilled off under reduced pressure at 60° C. The resulting brown sticky material was taken up in a small volume of benzene and centrifuged to remove insoluble material. The benzene solution was passed through an adsorption column of aluminium oxide, treated with HCl to bring it to pH 5. The active fraction from this column was passed through a second aluminium oxide column. On further passage through aluminium oxide, at pH 5, the material gave no signs of inhomogeneity. Repeated passage through aluminium oxide treated with CO_2, to bring it to pH 7, caused an increase in inhomogeneity of the material and a loss of total activity.

Further attempts to purify the active fraction from the second aluminium oxide, pH 5, column were unsuccessful, as judged by the following criteria. There was no change—

(1) In antibacterial activity per unit weight.

(2) In rotation of polarized light per unit weight.

(3) In the ratio of neutral to acid substance after alkaline or acid decomposition (see below).

Attempted crystallization was unsuccessful.

* With a grant from the Agricultural Research Council.

Physical and Chemical Properties.

Solubility.—Tardin is a pale yellow oil. It is only very slightly soluble in water. It is freely soluble in alcohol, acetone, ether, benzene, amyl acetate. It is slightly soluble in warm hexane. For cylinder-plate tests tardin was dissolved in acetone or aqueous acetone.

Interference.—When neat acetone was used as a solvent it was necessary to raise the lid of the assay plates in order to reduce the concentration of acetone vapour. Provided this was done, acetone did not interfere with the assay. For biological work a concentrated acetone solution of tardin was added to the aqueous medium and heated to 100° for 5 minutes to remove the acetone. By this means concentrations of 1 mg./ml. of tardin as a fine emulsion were prepared.

Stability.—Tardin is stable between pH 2 and 8, but above pH 8 is readily decomposed, yielding two fractions, an acid and a neutral substance. The proportion of the acid fraction increased during purification, the purest product obtained containing 85 per cent. On acid hydrolysis (N acid at 100° C.) tardin yields an acid and a neutral fraction in the same proportions (i.e. 85 per cent acid fraction).

Reducing power.—The acid formed by the alkaline breakdown of tardin reduces Fehling's solution.

2: 4-*Dinitrophenylhydrazine.*—Both the acid and neutral breakdown products of tardin react with 2: 4-dinitrophenylhydrazine to give an amorphous precipitate, but tardin itself does not react.

$FeCl_3$.—Tardin in acetone or alcohol gives a purple colour with $FeCl_3$, which goes brown on warming.

Test for $\beta\gamma$ unsaturated lactone.—The test described by Jacobs and Hoffmann (1926) was applied to tardin and was negative.

Optical activity.—The best preparation showed a slight laevo-rotation in alcohol. $D\frac{\alpha}{20} - 11{\cdot}4$.

Elementary analysis (Weiler and Strauss).—On elementary analysis tardin was found to have the following percentage composition: C 68·2, H 7·58, O 24·22. No other elements were present.

Calculated percentage composition for $C_{11}H_{15}O_3$: C 67·8, H 7·7, O 24·6.

Less pure preparations contained about 1·5 per cent nitrogen.

Antibacterial Action.

Inhibition of growth.

The inhibitory action of tardin was tested on a number of bacterial species by the serial dilution method (Table I). The tardin was suspended as a fine emulsion in sterile heart extract broth, which was then resterilized by standing in a boiling water bath for 5–6 minutes. Two-fold serial dilutions were made from this and measured in 5 ml. amounts into test-tubes. Each tube was inoculated by a Dreyer's pipette with 1 drop of an 18–20-hour broth culture diluted 1 in 1000, except with the cultures *Str. viridans* and *C. diphtheriae*, which were not diluted. Results were read after overnight incubation, by naked-eye comparison of the opacity of the tubes.

TABLE I.

	Highest dilution of tardin giving inhibition.		Lowest dilution giving no inhibition.
	Complete.	Partial.	
Staph. aureus	1 : 128,000	1 : 256,000	1 : 512,000
Str. viridans	1 : 8,000	1 : 16,000–1 : 64,000	1 : 128,000
C. diphtheriae gravis	1 : 8,000	1 : 16,000–1 : 32,000	1 : 64,000
C. xerosis	1 : 64,000	—	1 : 128,000
Salm. enteritidis	1 : 1,000	1 : 2,000	1 : 4,000
Bact. coli	—	—	1 : 1,000
Ps. pyocyanea	—	—	1 : 1,000

The table shows that tardin is more active against the Gram-positive than against the Gram-negative organisms tested. The relatively low titres against *Str. viridans* and *C. diphtheriae* may be due, at least in part, to partial inactivation of the tardin by the serum in the medium (see later).

Bactericidal action.

After overnight incubation of the dilution series a loopful was plated out from each completely or partially inhibited dilution tube. Where there was no visible growth in the dilution tube the plating was sterile, the only exception being *C. xerosis*, which gave a partial growth from the 1 in 64,000 tube ; the plating from 1 in 32,000 was sterile.

Effect of inoculum size.—Against *Staph. aureus* a thousand-fold reduction in the size of the inoculum made no difference to the end-point in the serial dilution test.

Inhibition by serum.

Incubation for 2 hours with 50 per cent serum at 37° reduced the activity of tardin (1 mg. per ml.) by 60 to 80 per cent. Incubation for 2 hours in 10 per cent. serum at 37° reduced the activity by 20 to 30 per cent.

Action against pathogenic moulds.

It was found that the growth of *Sabouraudites audounii*, *Trichophyton sabouraudii*, *Trichophyton equinus* and *Sabouraudites lanosus* was inhibited at 1/5000, while *T. lacticolor*, *T. tonsurans* and *Endomycopsis albicans* grew at that dilution. All the fungi grew in 1/20,000 tardin.

Pharmacology.

Toxicity to leucocytes.

The effect of tardin on body cells *in vitro* was tested by placing human leucocytes in contact with different concentrations of tardin, the method described by Abraham *et al.* (1941) being used. The tardin was in the form of a fine emulsion obtained by agitating the substance with the medium in which the leucocytes were examined.

In 1 in 16,000 the cells did not differ appreciably from those in control preparations. In 1 in 8000 many of the cells were active, but some died in the first hour. In 1 in 4000 all cells were dead in an hour, and in 1 in 2000 in half an hour. Duplicate tests agreed with these figures.

This relatively low toxicity to leucocytes may perhaps partly be due to the low solubility of the substance or to its partial inactivation by 10 per cent serum.

Toxicity to mice.

Mice weighing 20 g. were used. Owing to the insolubility of tardin the largest dose which could be given *intravenously* was 0·8 mg. in 0·5 c.c. of saline. After doses of from 0·36 to 0·8 mg. mice appeared slightly unwell, but recovered within an hour. No other effects were noted. *Subcutaneously* a dose of 1·8 mg. in 0·3 c.c. saline had no general effect, but 2.5 mg. in 0·4 c.c. produced transient sickness. A few hours later in both mice there was oedema at the site of injection and subsequently the skin sloughed.

Effect on smooth muscle.

The rhythmic contractions of the isolated guinea-pig uterus first increased in rate and then were abolished after 1 : 25,000 tardin. There was no recovery of the rhythm on washing with Ringer's solution. In one experiment rhythmic contractions were initiated by 1 : 50,000 in a quiescent uterus.

Effect on blood pressure of the cat.

The intravenous injections of 26 mg. of tardin as a fine emulsion in 26 c.c. of saline had no effect on the blood pressure of a cat weighing 3·4 kg. anaesthetized with chloralose. In another experiment on a cat weighing 2·5 kg. anaesthetized with nembutal there was a fall of blood pressure after the injection of 30 mg. in 30 c.c., followed by recovery within 3 minutes to the initial level.

Heart perfusion.

In 9 minutes 1/25,000 produced almost complete stoppage of the heart from which there was no recovery. Similar but proportionately more delayed effects were obtained with dilutions of 1/50,000, 1/100,000 and 1/200,000.

SUMMARY.

1. An antibacterial substance has been extracted from the culture fluid of *Penicillium tardum*. It has been called tardin.

2. Some of its chemical and biological properties are described.

3. Its properties are such as to make it unlikely that it has therapeutic possibilities.

REFERENCES.

Abraham, E. P., Chain, E., Fletcher, C. M., Florey, H. W., Gardner, A. D., Heatley, N. G., and Jennings, M. A.—(1941) *Lancet*, ii, 177.

Jacobs, W. A., and Hoffmann, A.—(1926) *J. biol. Chem.*, **67**, 333.

Wilkins, W. H., and Harris, G. C. M.—(1943) *Brit. J. exp. Path.*, **24**, 141.

We are much indebted to M. A. Jennings, A. G. Sanders, B. F. Pausaker, M. Lancaster and J. Kent for assistance with some of the experiments.

ANTIBIOTICS FROM *STEREUM HIRSUTUM*.

N. G. HEATLEY, M. A. JENNINGS AND H. W. FLOREY.

From the Sir William Dunn School of Pathology, Oxford.

Received for publication December 16, 1946.

THIS paper records observations on some antibacterial substances, and others chemically related to them, produced by a species of fungus which was first encountered as a chance contaminant on an agar plate. Some interesting features about its metabolic products have prompted the publication of these results. Briefly, the fungus liberates into the metabolism fluid a precursor, which has been called hirsutic acid C, which it converts at a certain stage in its growth to an antibacterially active substance (hirsutic acid N). This active substance is produced also *in vitro* on incubation of the precursor with the washed mycelium of the fungus or with an extract of mycelium. These two substances have been purified and the precursor has been crystallized. Two other antibacterial substances have been detected in the metabolism fluid, but have not appeared to be worth detailed investigation.

As well as the three formed naturally, at least two other antibacterial substances have been produced by chemical treatment of the precursor. One of these (hirsutic acid A) is formed when the precursor is treated with alkali. This also has been purified but not crystallized. An inactive acid which is formed on alkaline treatment of hirsutic acid C or hirsutic acid A completes the list.

The derivatives and some of the properties of the various substances are set out in Table I. The inhibitors are of low potency against bacteria, and are relatively toxic to tissue cells. Though much more work could be done on all aspects of these substances, the novel relationship which was demonstrated when an inactive extracellular precursor was shown to give rise to antibacterially active derivatives encouraged us to publish these incomplete examinations.

EXPERIMENTAL.

The Fungus.

The fungus was kindly examined by Dr. W. H. Wilkins, who considered it to be a strain of *Stereum hirsutum*. Mr. K. StG. Cartwright has recently examined the mould independently, and reported that though it had many characteristics of *S. hirsutum*, it differed from his reference strain in not developing the exact coloration of the latter; though he felt that this was within the variation which might be shown by different strains, there was the possibility that it might be an allied species.

A culture was deposited with the National Collection of Type Cultures, The Lister Institute, London, in 1942, where it was assigned the number 6475 and the provisional name *Stereum hirsutum*. The mycelium forms a freely growing white fluffy mass, easily propagated by mycelial transfer, but sporulation has not yet been induced in artificial culture.

Methods of Culture and Assay.

Good growth and good development of inhibitory power took place on 2 per cent malt extract, potato-dextrose or Sabouraud's medium, but Lemco broth, Czapek-Dox, or Czapek-Dox plus corn steep liquor were unsuitable. Two per cent malt extract in tap water was used throughout this work. When cultures were grown at 24° C. on a layer of this medium 1–2 cm. deep, antibacterial power made its appearance in the medium abruptly at the ninth or tenth day. At this time a sample of the brew gave a zone of inhibition 15 to 20 mm. in diameter on a cylinder-plate surface-seeded with an undiluted broth culture of *Staph. aureus*, and thereafter the titre remained unchanged for at least two weeks.

The cylinder-plate method (Heatley, 1944) has been used for assay purposes.

Preparation of Crude Extracts.

The nature and relative amounts of the products obtained depended to a great extent on the duration of incubation. Usually the culture fluid was harvested on the tenth or eleventh day, when the antibacterial titre had reached a maximum, but a few batches were left for double this time. The broth was acidified to pH 2·0 and extracted with amyl acetate. The partition coefficient was very favourable, and five volumes of medium were extracted with one of solvent. The amyl acetate extract was concentrated to a thin syrup by vacuum distillation and placed in the refrigerator for two days, when a sandy crystalline deposit formed. This substance, an acid which shows no antibacterial action, will be referred to as hirsutic acid C (" Crystalline ").

In dealing with metabolism fluid from older cultures, acid C was not deposited, but a gum was often precipitated instead. In either case the orange syrupy mother liquor was diluted with an equal volume of ether (to reduce the viscosity) and then passed through a short column of alumina (B.D.H. " For Chromatographic Analysis "), which was developed with further large quantities of ether. Some brown pigment (and gum if the medium was harvested late) remained on the column, but practically all of the active material passed through. The various percolates were combined, and sometimes this mixing of fractions which were individually quite clear resulted in the precipitation of a white gum. This was inactive, and was removed by centrifugation or filtration. The resulting clear yellowish-orange solution was concentrated 5 to 10 times by distillation at atmospheric pressure, and was then extracted several times with an equal volume of water of which the pH was maintained at 6·0–6·5 with sodium hydroxide. Much of the pigment and a small amount of active substance remained in the ether, but both could be removed quite easily by extracting with water at pH 8·0–9·0. The fact that most of the activity could be easily removed at pH 6·0 suggested that the residual inhibitor was a different compound. This residual inhibitor was not investigated further (" Unclassified 1 " in Table I).

Another inhibitor was formed in small amounts when the medium was harvested late, and could be separated by the fact that it was not readily extracted from benzene into water at any pH; it is therefore probably not an acid. This inhibitor when tested on a gutter-plate was

TABLE I.—*Products Obtained from* Stereum hirsutum.

Designation.	Origin.	Chemical characteristics.	Antibacterial activity.	Toxicity to leucocytes.	Toxicity to mice.
Hirsutic acid C	Metabolism fluid, up to 12th day and possibly later	Crystalline acid, $C_{15}H_{20}O_4$	Inactive	..	24 mg. without effect.
Hirsutic acid N	(*a*) Metabolism fluid after about 9 days. (*b*) Formed on incubation of acid C with washed mycelium or extract of mycelium	Non-crystalline acid	Active; titre for *Staph. aureus* about 1 in 10,000; activity differential	1 in 16,000 deleterious, 1 in 4000 lethal	3 mg. lethal.
Hirsutic acid A	Formed by treatment of acid C with dilute alkali	Non-crystalline acid; more stable to alkali than acid N	Active; titre for *Staph. aureus* about 1 in 8000; activity differential	1 in 16,000 deleterious, 1 in 4000 lethal	20 mg. lethal.
Hirsutic acid X	Formed by action of alkali on acid A or acid C	Non-crystalline acid	Inactive	..	9 mg. without effect.
Unclassified 1	Metabolism fluid young cultures (small amounts)	Acid with higher pK than C, N, A or X	Active	..	..
Unclassified 2	Metabolism fluid, old cultures (small amounts)	Probably not an acid	Active; activity not differential	..	..
Unclassified 3	Formed by action of dry heat on acid C	An acid; possibly the same as acid A	Weakly active	..	.
Unclassified 4	Formed by action of acid on acid C	Not an acid	Weakly active	..	..

found to be about equally effective against all of the few kinds of bacteria tested. It occurs in extremely small yield, and this substance also was not investigated in greater detail (" Unclassified 2 " in Table I).

The successive aqueous extracts made at pH 6·0 from the concentrated ether were pooled, acidified to pH 2·0 (which produced a dense cream-coloured precipitate or emulsion), and extracted with ether, the weakest extracts being treated first. Here again the partition coefficient was very favourable to the solvent.

The combined ether extracts were passed through a column of silica intimately mixed with about three-fifths of its weight of strong phosphate buffer pH 7·0 (Martin and Synge, 1941), and were followed by copious washings of ether. Usually a bright yellow pigment without antibacterial power was left in the upper part of the column, sometimes with a narrow zone of brown pigment on top of it; in a few batches both brown and yellow pigments were absent. The ether percolates, which contained the whole of the activity, were concentrated by distillation and extracted into water at pH 6·5–7·0. The aqueous extracts were then shaken with that amount of Farnell " Grade 14 " charcoal which by preliminary tests with an aliquot portion were found to remove most of the pigment but practically no activity. The filtrate was again acidified and extracted with ether, which was passed through a column of alumina and developed with more ether. The remaining pigment stayed at the top of the column, but almost all of the activity was confined to a zone situated near the bottom of the column which gave a very faint purple fluorescence in ultraviolet light. This fraction was eluted with phosphate buffer, from which, after acidification, the inhibitor was taken into ether. This last extract was completely colourless, and on evaporation left a clear transparent glaze which appeared to retain its activity indefinitely. This inhibitor will be referred to as hirsutic acid N (" *N*aturally-occurring ").

Chemical Investigations.

Hirsutic acid C, the antibacterially inactive precursor.

Obtained in crude form from the concentrated amyl acetate extract of the medium, this substance can be recrystallized from alcohol or aqueous alcohol. Crystallization always proceeds slowly and, even if a cooled supersaturated solution is seeded, several hours are required for the complete separation of the solid. The material crystallizes in large colourless prisms which can be obtained a centimetre or more in length without difficulty. They contain no water or alcohol of crystallization. Dr. M. W. Porter recrystallized and examined these and reported as follows:

> " The crystals belong to the rhombohedral system. They are both single and twinned. The forms observed on excellent single crystals, which are prismatic in habit and doubly terminated, are as follows: $r(100)$, $a(10\bar{1})$, $m(2\bar{1}\bar{1})$, and sometimes the form $c(111)$, which is matt. The Barker classification angle cr $(111 : 100) = 43° 45'$.
>
> " The twinning is about the c-axis, and the crystals show the same forms as described above. Optically the crystals are positive."

The crystals, which are odourless and almost tasteless, are sparingly soluble in cold water (about 1·5 mg./ml. at room temperature), more soluble in hot

water and soluble in most organic solvents except hexane and carbon tetrachloride. The barium salt is freely soluble in water. The free acid in absolute alcohol is dextrorotatory, having $[\alpha]_D^{20^\circ} = +11{\cdot}9^\circ$.

Nitrogen, sulphur, phosphorus and halogens are absent, and elementary analysis (Weiler and Strauss) gave C, 68·3 per cent; H, 7·47 per cent. The material titrates as a monobasic acid with a pK of about 4·6 and an equivalent weight of 260–264. The formula $C_{15}H_{20}O_4$ has C, 68·2 per cent; H, 7·57 per cent, mol. wt. 264, which fits the observed values fairly well. The mol. wt. has been confirmed by X-ray crystallographic methods. Mrs. A. Joseph and Mrs. B. W. Rogers, who carried out this work, gave the following brief report:

> "The crystals described by Dr. Porter above gave good X-ray photographs from which the following data were obtained: $a = 11{\cdot}4$ A°, $c = 9{\cdot}5$ A°, Space Group $C3_1$ $n = 3$. The density by flotation was found to be 1·238. From these values the calculated molecular weight 268 ± 6 was obtained, which is in good agreement with the calculated molecular weight 264 for the formula $C_{15}H_{20}O_4$.
>
> It is of interest to note that the crystals were found to be strongly pyro-electric."

The substance does not fluoresce appreciably, and has no ultra-violet absorption at wavelengths longer than 230 Ångstrom units; Dr. Holiday, who made this last observation, stated that in view of the absence of absorption it was unlikely that the compound could contain a pair of conjugated double bonds. The substance melts at 179·5° C. (uncorr.). It does not reduce Fehling's solution or ammoniacal silver nitrate, does not react with 2·4-dinitrophenylhydrazine, and does not liberate free iodine from acidified potassium iodide. It decolorizes potassium permanganate very slowly, but reacts at once with bromine water, giving a white precipitate. With ferric chloride it gives a faint brown colour. Methoxyl groups are absent (Weiler and Strauss), and hydroxyl groups estimated by the method of Freed and Wynne (1936) are also absent, though a positive nitro-chromic reaction (Fearon and Mitchell, 1932) is obtained. The substance appears to have one easily hydrogenated double bond.

It reacts in ether solution with diazomethane to give orthorhombic plates or needles m.p. 161–162° C. (uncorr.). These contain C, 68·8 per cent; H, 7·82 per cent; mol. wt. by Rast method, 283 (Weiler and Strauss). If a simple esterification occurs, the expected methyl ester $C_{16}H_{22}O_4$ would contain C, 69·1 per cent; H, 7·92; mol. wt., 278. This derivative is too insoluble in water for its possible antibacterial effect to be examined.

Hirsutic acid N, the naturally-occurring antibacterial substance.

This is obtained from the crude culture fluid by the method outlined above, and appears as a transparent, colourless glaze, almost insoluble in cold water, slightly soluble in hot water and readily soluble in alkali. It is soluble in most organic solvents except hexane and carbon tetrachloride. It has no sharp melting-point, but when heated on a slide it bubbles slightly and gives off pungent vapours which are extremely irritating to the nose and throat. Many attempts were made to obtain the substance in crystalline form, or to obtain a crystalline derivative, but without success. The substance titrates as a monobasic acid

with a pK close to that of acid C, i.e. 4·6. The substance may be distilled in a high vacuum at about 125° C., and though no activity appears to be lost after two hours at this temperature, little if any purification takes place, and the distillate is amorphous.

The free acid, either solid or in ethereal solution, appears to be stable indefinitely, but an aqueous solution of the sodium salt when incubated at 37° C. at pH 6·5–7·0 has a half life of slightly over two days; at room temperature (20° C.) the rate of loss of activity is correspondingly less. At pH 9·0 some activity is lost after 30 minutes at room temperature; in the presence of N/10 sodium hydroxide, half the activity is lost in five minutes at room temperature. The material is much more stable in the presence of aqueous acid, losing less than half its activity when heated to 100° C. for five minutes in the presence of 0·3 N hydrochloric acid.

Hirsutic acid N produced by incubating washed mycelial mats with the sodium salt of hirsutic acid C.

If the mycelium of the mould at the time when it has reached the stage of forming an inhibitor is well washed with distilled water and then floated on a solution of the sodium salt of acid C, activity gradually appears, reaches a peak, then falls. In one experiment at 37° C., with a layer of solution a few mm. thick containing 5 mg./ml. of neutralized hirsutic acid C, the peak was reached after about 5 hours; at room temperature the peak was reached more slowly, but had declined to considerably less than half its value after 30 hours. This property of the mycelium is destroyed by brief boiling. On grinding it with sand and centrifuging, the supernatant liquid is found to possess the same power as the intact mould mat; it is not affected by the presence of chloroform. The mould mats retain this property after storage for at least 6 months at + 4° C. This inhibitor appears to be identical with the naturally-produced hirsutic acid N described above, which is only to be expected in view of its relation to the time sequence of events when the mould is growing naturally, for the mycelium does not acquire the property of converting acid C to acid N *in vitro* until the time at which antibiotic activity appears in the medium. The accumulation of acid C in the medium just prior to this time accords with this line of reasoning.

Solutions of the sodium salt of acid C were incubated with the sterile washed mats of several species of *Penicillia*, some *Aspergilli*, and one *Basidiomycete* (*Tricholoma nudum*), but in no case was an inhibitor produced.

Acid A, an active substance derived from acid C by treatment with alkali, and acid X, an inactivated product.

Early during the work an attempt was made to recover hirsutic acid C from crude concentrates containing only small amounts, by converting it to the methyl ester with diazomethane, isolating and purifying this, and then regenerating the free acid by alkaline hydrolysis. This led to the discovery that when acid C was warmed with very dilute (0·01 N) NaOH, or allowed to stand at room temperature with stronger alkali, a bacterial inhibitor was first formed and then slowly destroyed. Thus, at 20° C. in the presence of N/10 sodium hydroxide, the maximum titre was reached in about 2 hours, but no activity could be detected at 30 hours (Fig. 1). Whatever the conditions, it seems

that the two reactions—activation and destruction—proceed at the same time, so as to produce a mixture of the active and inactive products, known respectively as hirsutic acid A ("*A*lkali-activated") and hirsutic acid X, sometimes together with unchanged hirsutic acid C. All three substances are acids with pK values close to 4·6, and this, together with the failure of all attempts to crystallize or to obtain crystalline derivatives of the active substance and of attempts to separate the mixture, has hindered investigation. Some purification, however,

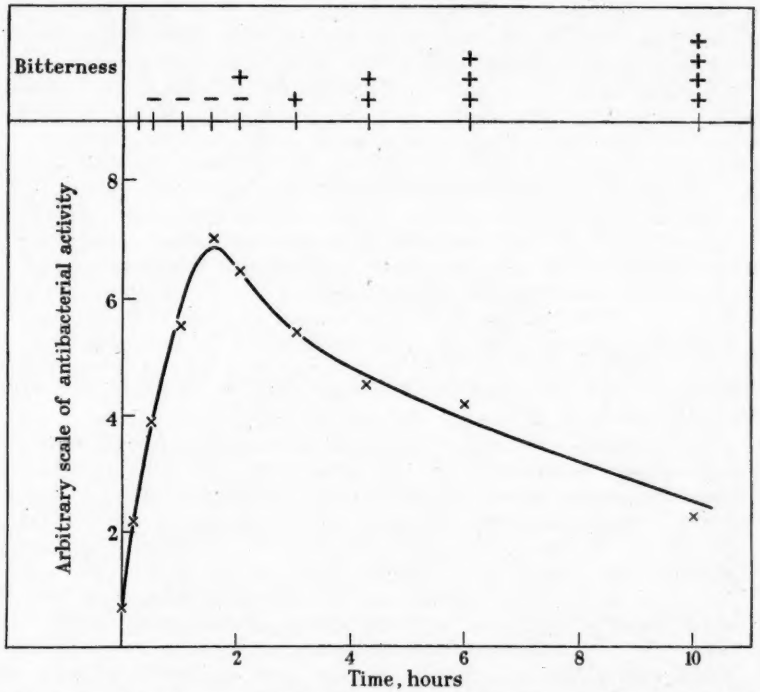


FIG. 1.—Course of development of antibacterial activity and bitterness on incubating hirsutic acid C with N/10 sodium hydroxide at 20° C.

has been effected by passing an ether solution of the mixed acids through an alumina column, when acid A is found in the lowest part, unchanged acid C next, and the inactivated acid X above this, with small amounts of pigment at the top of the column. The coloured layer is yellow in acid, pink in alkaline solution.

The activation of hirsutic acid C by alkali and the subsequent destruction of antibacterial power is not accompanied by the liberation of carbon dioxide, nor is there a change in the titratable acid groups. As the action of alkali on acid C proceeds, a bitter taste develops, which, because it continues to increase after the peak of antibiotic value is passed, must be due to the inactive product hirsutic acid X, not to the inhibitor hirsutic acid A (Fig. 1). Increase in

bitterness is paralleled by decreasing nitrochromic reaction until the latter eventually becomes completely negative, though the heavy precipitate, which forms on adding the reagent and then dissolves, persists.

The best yields of acid A are obtained by keeping a solution of up to 100 mg. of acid C per ml. in a final concentration of approximately N/10 NaOH at 20–24° C. for 30 minutes, then acidifying to pH 2·0 and leaving in the refrigerator overnight. The crop of crystals of unchanged acid C are filtered off and treated with N/10 alkali as before. The filtrate containing the inhibitor in solution is pooled with the subsequent filtrates and extracted with ether while still acid. The pooled ether extracts are then purified by alumina chromatography.

When an ethereal solution of the free acid A is evaporated and dried a colourless brittle glaze is obtained. All attempts to obtain the substance in crystalline form were unsuccessful, and the purest preparations probably contained some of the alkali-inactivated acid X and/or unchanged acid C. The solubility of the glaze seems to be qualitatively similar to that of acid C and acid N. It does not reduce Fehling's solution or ammoniacal $AgNO_3$, iodine is not liberated from acidified KI, bromine water is slowly decolorized, giving a white precipitate soluble in NaOH, and $KMnO_4$ is also decolorized slowly, but these effects may be due to the presence of acid C and of acid X respectively. Activity is destroyed on reducing with the Al-Hg couple.

Comparison of hirsutic acids N and A.

That the acids N and A are definitely different is shown by the following characteristics :

(1) Acid N, the natural inhibitor, is much more sensitive to alkali, e.g. at room temperature, in the presence of N/10 alkali, its half life is about 5 minutes, whereas under identical conditions acid A, the alkali-formed substance, shows no appreciable destruction after 30 minutes. At pH 7·0 the former has a half life at 37° C. of about 2 days, the latter of more than 2 weeks.

(2) In the cylinder-plate assay method, the natural inhibitor gives sharp zones of inhibition, and the zone diameter-concentration curve is rather flat ; the alkali-formed inhibitor on the other hand usually gives zones with a rather indefinite margin, and the curve is much steeper over any given range of zone size. (This may be related to the difference in pH stability.)

Other methods of activating hirsutic acid C.

Inhibitor formed by the action of dry heat on hirsutic acid C (" *Unclassified* 3 " *in Table* I).—When 2 mg. samples of acid C were heated to 230° C. for varying lengths of time from 2 to 10 minutes, the melt, dissolved in a little alcohol and water and brought to neutral pH in a final volume of 1 ml., gave a definite, though small and ill-defined, zone of inhibition on a cylinder-plate seeded with undiluted *Staph. aureus* broth culture. At 265° C. activity was produced after 1 minute, but diminished if heating was prolonged, being scarcely detectable after 5 minutes. At this temperature the melt became brownish, and a ring of condensed droplets formed in the cooler part of the tube. The activity, however, remained in the body of the melt, the condensate being inactive.

This inhibitor could be extracted into ether from acidified watery solution and extracted from the ether into dilute phosphate buffer. In pH stability it resembled the inhibitor formed by the action of alkali. The production of the inhibitor was not accompanied by a change in titratable acid groups.

Activation of hirsutic acid C by acid hydrolysis ("*Unclassified* 4" *in Table* I). If crystals of hirsutic acid C were refluxed with N/100 HCl for 1 to 2 hours, the unchanged hirsutic acid C could be extracted with ether, but the neutralized aqueous residue gave small and not very sharp zones of inhibition on an assay plate seeded with *Staph. aureus*. This inhibitor is apparently not an acid, and thus differs from the other inhibitors investigated; it is either produced in poor yield or is a not very powerful antibiotic, and no further work was done on it.

BIOLOGICAL INVESTIGATIONS.

1. *Antibacterial Activity.*

Hirsutic acid N.

Serial dilution tests were done in heart extract broth in two- or four-fold series. After incubation the presence or absence of growth was determined by naked-eye inspection. In a four-fold series the highest titre for complete inhibition of growth of *Staph. aureus* and *Str. pyogenes* was 1 in 10,000, and of *C. diphtheriae* and *N. meningitidis* 1 in 40,000. Comparative titrations in two-fold series with less pure material showed that *Staph. aureus* was slightly more sensitive than *Str. pyogenes*, and that *Bact. coli* and *Salm. enteritidis* were relatively insensitive, the end-point for these organisms not being reached.

In addition to the quantitative tests, *gutter-plates* were made in which acid N was incorporated with the agar in the gutter. The plates were cross-streaked with several organisms and incubated overnight. From the amount of inhibition of the streaks the organisms could be arranged in decreasing order of sensitivity as follows:

Micrococcus lysodeikticus,
B. anthracis,
Sarcina lutea,
Staph. aureus,
El Tor vibrio
B. subtilis.

The following were not inhibited: *Salm. typhi* (H), *Bact. coli*, *Bact. aerogenes*, *Bact. friedländeri*, *Proteus*, *Ps. pyocyanea*, and *Chr. prodigiosum*.

Effect of inoculum size.—In two-fold dilution series a thousand-fold difference in the size of the inoculum of *Staph. aureus* made no difference in the titre.

Hirsutic acid A.

The highest titre obtained in *serial dilution tests* against *Staph. aureus* was 1 in 8000. In a comparative test with less pure material *Staph. aureus* was shown to be slightly more sensitive than *Str. pyogenes*, and *Bact. coli* and *Salm. enteritidis* to be less sensitive, the end-point for these organisms not being reached.

Effect of inoculum size.—A 2500-fold alteration in the size of the inoculum had no effect on the titre against *Staph. aureus*.

Action on the staphylococcus.—A growing culture of *Staph. aureus* was incubated at 37° C. in a water-bath for 24 hours in the presence of 0·2 per cent of hirsutic acid A. There was no visible lysis of the cocci.

Effect of serum and peptone.—Solutions of hirsutic acid A in water and in various percentages of ox serum (up to 50 per cent) and of peptone (up to 5 per cent) were assayed on cylinder-plates. In no case was there a significant difference between the respective assay values.

2. *Toxicity to Leucocytes.*

Hirsutic acids N and A.

The effect on human leucocytes was examined by the method previously described (Abraham, Chain, Fletcher, Florey, Gardner, Heatley and Jennings, 1941). The concentrations which were lethal to leucocytes were of the same order as those which stopped the growth of the staphylococcus, and were the same for both substances. Using material which had one-quarter to one-eighth of the antibacterial activity of the purest material, a 1 in 4000 solution killed or immobilized most of the leucocytes immediately, though a few moved sluggishly for about an hour. In a 1 in 16,000 solution the leucocytes lived somewhat longer, and in a 1 in 64,000 solution they were indistinguishable from those in control preparations.

3. *Toxicity to Mice.*

The material was administered to mice weighing from 20 to 22 gm. intravenously and (in the case of the natural substance) by injection into the stomach.

Hirsutic acid N.

The maximum dose of the sodium salt which was tolerated intravenously was between 1 and 2 mg.; 6 mg. killed in 5 minutes, and intermediate doses in from a few minutes to 2 or 3 hours. When given by mouth 20 mg. had no effect, and it is possible that the substance either was not absorbed from, or was destroyed in, the gastro-intestinal tract, as no antibacterial activity was found in the urine.

Hirsutic acid A.

Fifteen milligrams given intravenously caused severe transient sickness but recovery was complete. After 20 mg. a mouse remained sick for 48 hours and then died.

Hirsutic acid C.

This bacteriologically inert material was apparently non-toxic, for 12, 16, 18 and 24 mg. of the sodium salt injected intravenously (into larger mice, weighing up to 31 gm.) did not give rise to any toxic symptoms. With the idea that the inactive precursor might be activated in passing through the body the urine of these mice was collected and tested. No activity could be detected by the cylinder-plate method.

Hirsutic acid X.

After intravenous doses of 5 and 9 mg. respectively, two mice remained perfectly well.

4. *Effect on Blood Pressure and Respiration.*

Hirsutic acid N.

Fifty mg. in 2·5 c.c. of saline injected intravenously into a cat weighing 3·4 kg. anaesthetized with nembutal induced a rise of blood pressure from 132 mm. Hg to 150 mm. Hg. After 25 minutes the level was 144 mm. Hg, and the further injection of 44 mg. raised the pressure momentarily to 192 mm. Hg, after which a pressure of 174 mm. Hg was maintained for at least 45 minutes.

In another cat under nembutal which had an initial pressure of 160 mm. Hg the injection of 100 mg. momentarily raised the pressure to 180 mm. Hg, after which it fell back to 164 mm. Hg and was maintained there.

Thus it appears that this substance will raise the blood pressure, but the mechanism by which this rise is brought about has not been further investigated. The respiration rate was not altered in either animal.

Hirsutic acid A.

Twenty-five mg. in 2 c.c. of saline were injected intravenously into a cat under chloralose anaesthesia, and were followed 7 minutes later by 100 mg. There was no significant effect on the blood pressure, heart rate or respiration rate from these injections.

5. *Effect on the Perfused Heart.*

Hirsutic acid N.

Perfusion of the isolated cat's heart with acid N at a concentration of 1 in 25,000 caused slowing of the heart, relaxation of tone and diminution of amplitude, the effect being manifest after a few minutes and progressing until at the end of from 60 to 80 minutes the contractions were only just perceptible. Recovery did not occur on perfusing with Ringer's solution. Perfusion with a 1 in 100,000 solution produced similar effects, but to a much slighter degree.

Hirsutic acid A.

A rabbit heart was perfused continuously with a 1 in 20,000 solution for 4 minutes without producing any effect. During perfusion for about the same time with 1 in 10,000 and 1 in 5000 solutions there was a slight decrease in amplitude, rate and coronary flow, followed by complete recovery on resuming perfusion with Ringer's solution.

6. *Effect on Smooth Muscle.*

Hirsutic acid N.

Immersion of the rhythmically contracting guinea-pig uterus in 1 in 5000 of acid N in Ringer's solution produced cessation of the rhythmic contractions; the uterus usually remained completely relaxed, but in one experiment tonic contraction occurred. In two experiments stimulation of the amplitude of the contractions was observed in 1 in 10,000 and 1 in 25,000 solutions, but this result was not obtained in 4 other experiments.

Thus there was no specific effect on uterine contraction.

SUMMARY.

1. The production and methods of extraction of antibacterial substances from *Stereum hirsutum* are described.

2. The fungus liberates into the metabolism fluid an inactive extracellular precursor which is activated by an enzyme contained in the mycelium.

3. The inactive precursor can be converted to weakly antibacterial substances, different from the natural antibiotic, by chemical means.

4. Some of the chemical and biological properties of the substances are described.

5. The substances do not appear to have any therapeutic potentialities.

We are indebted to Dr. A. G. Sanders for some of the heart perfusion experiments. We are glad to acknowledge the technical assistance of J. Kent, M. Lancaster and P. Morris.

We have received financial assistance from the Medical Research Council and the Rockefeller Foundation.

REFERENCES.

ABRAHAM, E. P., CHAIN, E., FLETCHER, C. M., FLOREY, H. W., GARDNER, A. D., HEATLEY, N. G., AND JENNINGS, M. A.—(1941) *Lancet*, ii, 177.
FEARON, W. R., AND MITCHELL, D. M.—(1932) *Analyst*, **57**, 372.
FREED, M., AND WYNNE, A. M.—(1936) *Ind. eng. Chem.* (*anal.*), **8**, 278.
HEATLEY, N. G.—(1944) *Biochem. J.*, **38**, 61.
MARTIN, A. J. P., AND SYNGE, R. L. M.—(1941) *Ibid.*, **35**, 1358.

STUDIES ON EXPERIMENTAL GOITRE. VIII: THYROID TUMOURS IN RATS TREATED WITH THIOUREA.

H. D. PURVES AND W. E. GRIESBACH.

From the Thyroid Research Department of the New Zealand Medical Research Council, University of Otago, Dunedin, N.Z.

Received for publication January 7, 1947.

SINCE Bielschowsky (1944) demonstrated a synergistic action between the carcinogen 2-acetamidofluorene and the goitrogenic agent allylthiourea in the production of thyroid neoplasms, interest has been aroused in the role of the goitrogenic agents in inducing thyroid neoplasia. Griesbach, Kennedy and Purves (1945) showed that the administration of a rape seed diet which contains a goitrogenic agent produced thyroid neoplasms in the rat, and considered that the induction of these tumours was due to the long-continued stimulation of the thyroid by the thyrotropic hormone, and not to any specific neoplasia inducing effect of any substance in the rape seed. Bielschowsky (1945) has reported the production of adenomata in the rat thyroid by the administration of the goitrogenic substance allylthiourea alone, though the incidence of such tumours was

small compared with those produced by the combination of allylthiourea and acetamidofluorene. He considered on the basis of histological appearance that these adenomata were not under the same control by the thyrotropic hormone as is normal thyroid tissue.

The present communication reports the result of long-term administration of the goitrogenic substance thiourea. The nature of the thyroid tumours produced and the response of such tumours to fluctuations in thyrotropic stimulation are described.

METHODS.

Some of the rats used were of the local albino strain of *Rattus norvegicus* used in previous experiments. In addition a group of rats of a recent Wistar strain were used. The diet was that previously described as diet S 3 (Griesbach, Kennedy and Purves, 1945). Thiourea was administered as a 0·25 per cent solution in the drinking water. When it was desired to inhibit the thyrotropic hormone secretion, injections of thyroid extract were given without interrupting the thiourea administration. For the administration of the thyroid extract, thyroid powder (B.P. '32) was brought into solution by peptic digestion in 0·2 N HCl. The solution was then neutralized and diluted till 1 ml. contained 50 μg. of iodine. Injections of 0·2 ml. of this solution were given daily.

The thyroids were dissected out at autopsy, weighed and fixed in formol-saline. Serial sections were cut at 5μ and stained with Harris's haematoxylin and eosin or with Heidenhain's azan method. When adenomata were observed sections were taken at 2·5μ for the purpose of photomicrography.

TABLE I.—*Production of Thyroid Neoplasms by Long-term Thiourea Administration.*

No. of rat.	Experiment A (Males, local strain).		Experiment B (Females, local strain).		Experiment C (Males, Wistar strain).	
	Thiourea treatment (months).	Thyroid neoplasms.	Thiourea treatment (months).	Thyroid neoplasms.	Thiourea treatment (months).	Thyroid neoplasms.
1	11	nil	5	nil	6½	nil
2	16	A	9½	nil	8½	nil
3	16	A	12	A	12	A
4	17	A	12	A	16	A
5	17	A	19½	A	16	A
6	20½	A, C	20	A, C	17	A
7	22½	nil	22½	A, C, F	17	A, F
8	22½	C	23½	A, C, F	21	A
9	23½	A, C, F	23½	A	21	nil
10	23½	A, C	23½	A	21½	nil

A = Adenoma, benign histological appearance.
C = Carcinoma, malignant histological appearance evidenced by invasion of blood vessels.
F = Foetal adenoma.

RESULTS.

Thyroid tumours were observed in a high proportion of the rats which received thiourea for 12 months or longer. Histologically three classes of tumours were found—benign adenomata, adenocarcinomata and foetal adenomata. The incidence of the three types of tumours is summarized in Table I.

I. Production of thyroid adenomata by thiourea treatment.

(*a*) Ten male rats of the local strain were placed on thiourea at the age of 8 weeks. One of these died from an intracranial abscess 11 months from the commencement of the experiment. No adenomata were seen in the thyroid of this animal. The remaining nine animals were sacrificed at times ranging from 16 to 23½ months after the commencement of the experiment. In one of these no thyroid neoplasm could be found on histological examination. In the remaining eight animals one or more thyroid tumours were found. In one animal (No. 8 of Exp. A, Table I) the single neoplasm found was relatively malignant in histological appearance. In three other animals tumours of relatively benign and relatively malign appearance were coexistent. In the remaining four animals only benign looking tumours were observed.

(*b*) Ten female rats of the local strain were placed on thiourea at the age of 8 weeks. Two which died at the age of 5 and 9½ months had no thyroid tumours. The remaining eight animals were killed at various times after 12 to 23½ months' thiourea treatment. All eight had benign looking thyroid neoplasms detected by histological examination. Three of these rats had, in addition, malignant looking thyroid tumours.

(*c*) Ten male rats of a recent Wistar strain were placed on thiourea at the age of 8 weeks. In two which died after 6½ and 8½ months of thiourea treatment no tumours were found on histological examination. Six of the remaining eight animals had benign looking adenomata. No tumours were found in two animals killed at 21 months after the experiment had been started. None of the growths in the six tumour-carrying animals was classified as malignant.

II. Malignancy.

Up to 19½ months from the beginning of the experiment the tumours were similar in appearance to those previously seen and described by us in rats on Brassica seed diet (Griesbach, Kennedy and Purves, 1945). These tumours were benign in histological appearance. At the 20th month one rat was found to have a benign looking tumour in one thyroid lobe, while in the other lobe there was a tumour with histological evidence of malignancy. Of the thirteen animals which were killed after 20 months' or more thiourea treatment, three were without thyroid tumours. In the ten tumour-bearing rats seven tumours were found with frankly carcinomatous histological appearance. The main criterion of malignancy was invasion of the walls of blood vessels. In two of these animals the malignancy of the tumours was confirmed by the finding of metastases in the lungs. The appearances in these animals have been described in a previous publication (Purves and Griesbach, 1946).

III. Foetal adenoma.

In three instances nodules of solid cellular growth were found, which we consider to correspond closely to what is called "foetal adenoma" in human pathology. One of these nodules grew in the centre of a benign looking adenoma (Fig. 11 and 12), while each of the remaining two nodules, one of which has been mentioned in an earlier paper (Purves and Griesbach, 1946), appeared within an adenocarcinoma of the thyroid (Fig. 10). In every case the boundaries between the two kinds of tissue were distinct. The foetal adenoma nodules consisted of

solid masses of cells with large vesicular nuclei, poorer in chromatin than the surrounding tissue. There was a trabecular structure recognizable in one of these tumours, but no colloid formation could be seen, not even after three weeks of thyroid digest injections. Mitotic figures were observed in these foetal adenomata. No tendency to invade blood vessels was apparent, and no lung metastases of " foetal adenoma " structure were found.

IV. Response of thyroid tumours to thyrotropic hormone.

In order to study the response of the thyroid adenomata to the thyrotropic hormone, an experiment was made in which injections of thyroid active material were made to inhibit the secretion of thyrotropic hormone by the pituitary. The thyroid preparation used was a peptic digest of standard Thyroid B.P. '32, the preparation of which is described above. The injections of 0·2 ml. daily contained 10 micrograms of iodine per injection, which would contain about 5 micrograms of l-thyroxine. This is in excess of the daily requirement of the rat, and causes inhibition of thyrotropic secretion by the pituitary (Griesbach and Purves, 1945). The administration of the thiourea in the drinking water was continued throughout the experiment. Eight rats were used, four being chosen for the purpose from each of the experimental groups A and C, which at this time had received 16 months' thiourea treatment. Four rats were killed after 21 days of thyroid digest injections. The thyroid digest administration was then stopped, and the remaining four rats killed after a further 28 days' treatment with thiourea alone. All eight rats were found at autopsy to have extensive thyroid adenomata. The histological appearance of the adenomata in the four rats killed after 21 days of thyroid administration was profoundly altered compared with that of tumours before thyroid administration. The large spaces which, as we have pointed out, are formed by the coalescence of acini, were distended with dense, deeply-staining colloid forming large colloid lakes, quite unlike any appearance of the normal rat's thyroid. The epithelium of these adenomata was markedly inactive compared with the appearance of any of the adenomata of this series before thyroid administration, or of any of those previously described as occurring during rape seed administration. One of these adenomata, illustrated in Fig. 7 and 8, showed in places an extreme degree of flattening of the epithelium. In another adenoma the epithelium was cuboidal in structure, but differed from the cuboidal epithelium of normal thyroid activity in that the cytoplasm was extremely scanty and the nuclei were closely packed together (Fig. 5 and 6). This close packing of the cells, which is a characteristic of the adenomata before thyroid treatment (Fig. 2 and 4), does not allow of flattening of the epithelium except where distension by colloid accumulation has stretched the acinar walls and allowed space for lateral expansion of the cells (Fig. 8). In the four rats which received thiourea alone for 28 days after cessation of thyroid injections, the thyroid adenomata, like the surrounding normal thyroid tissue, showed histological evidence of reactivation. The cells of the adenomata were hypertrophic, as shown in Fig. 9, and were quite as active in this respect as the cells of thyroid adenomata before thyroid treatment. Colloid, however, remained in considerable amount in these reactivated tumours, and was clearly in excess of that present in thyroid adenomata which had not been subjected to the inactivating influence of thyroid injections. It was, however, distinctly thinner

than in those tumours obtained immediately after the 21 days' thyroid treatment, so that colloid absorption was evidently taking place, although at a slower rate than in normal thyroid tissue.

After 22½ months of thiourea administration the remaining five surviving rats were treated with thyroid digest by injection for a period of 3 weeks. When these animals were killed, two were found to have the benign looking type of adenoma in the thyroid, and these tumours were involuted, with colloid storage and flattened epithelium. The other three animals showed thyroid tumours classified as malignant on the basis of histology. The cells of these adenocarcinomata were involuted by comparison with those of any of the non-thyroid-treated tumours. The nuclei were small and pycnotic and the cytoplasm was very scanty. In certain areas acinar spaces filled with dense colloid were present, and in these areas the appearance was typical of the involuted benign adenoma. In other areas the epithelium was arranged in cords or trabecules without acinar formation, and in these spaces there was no colloid accumulation. The lung metastases found in one of these animals contained well-formed acini which were distended with colloid.

DESCRIPTION OF PLATES.

FIG. 1.—Thyroid of rat after 16 months' thiourea treatment. A single adenoma with cystic spaces containing dilute colloid. A small amount of compressed normal thyroid tissue surrounds the adenoma. H. & E., × 34.

FIG. 2.—High-power view of adenoma in Fig. 1. The tall columnar cells are clearly shown. The acinar space contains a small amount of coagulated colloid. H. & E., × 650.

FIG. 3.—Thyroid of rat after 16 months' thiourea treatment. The adenoma is highly cellular, and stains more deeply than the surrounding normal tissue. H. & E., × 19.

FIG. 4.—High-power view of part of adenoma in Fig. 3. In the upper right-hand corner is a blood-filled space lined by endothelium only. The cells are mainly columnar, and the acinar spaces are empty. H. & E., × 650.

FIG. 5.—Thyroid of rat after 16 months' thiourea treatment, followed by 3 weeks' treatment with thyroid digest. The cystic spaces are full of dense colloid. Colloid accumulation can be seen in the acini of the adjacent thyroid tissue. Before thyroid treatment this adenoma was probably similar to that of Fig. 1. H. & E., × 30.

FIG. 6.—High-power view of part of Fig. 5. The involution of the cells is evident by their scanty cytoplasm in comparison with Fig. 3. The close spacing prevents any further flattening of the cells. H. & E., × 270.

FIG. 7.—Thyroid of rat after 16 months' thiourea treatment, followed by 3 weeks' treatment with thyroid digest. An adenoma is shown with acinar spaces greatly distended by colloid accumulation. The dark mass in the lower right corner is the parathyroid. H. &. E., × 30.

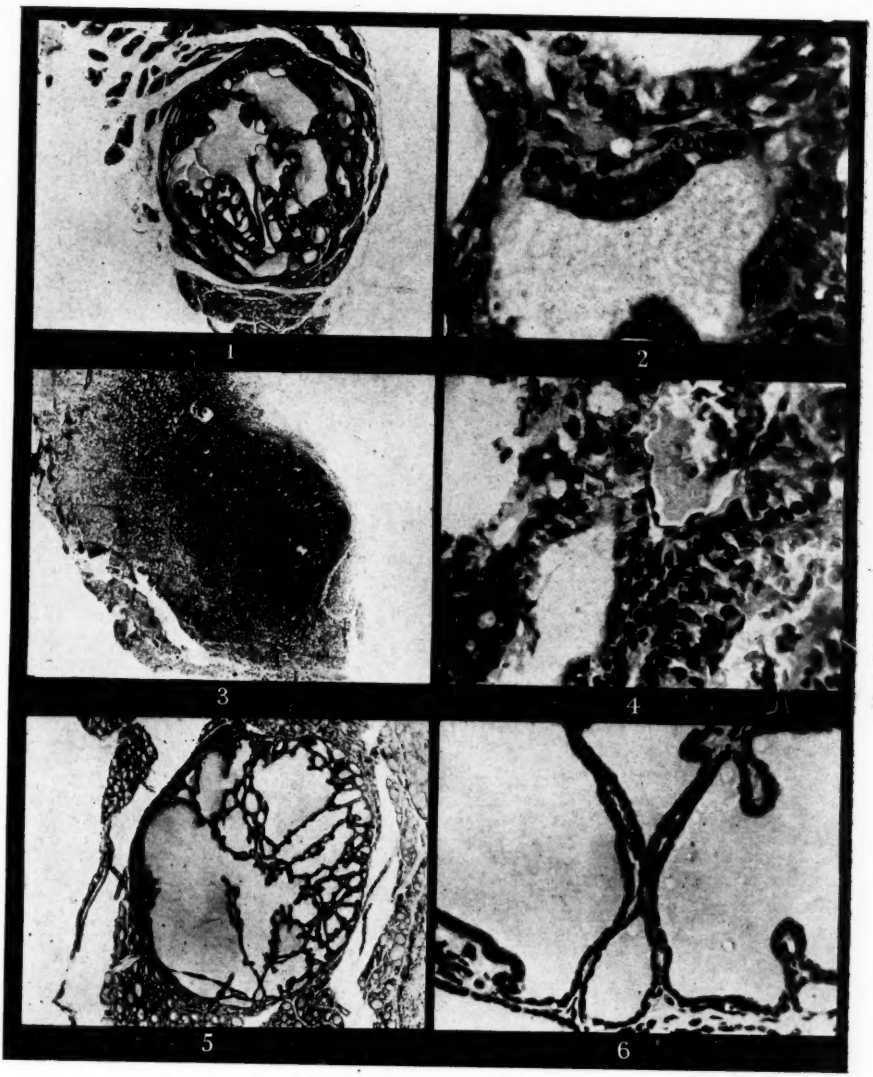
FIG. 8.—High-power view of part of Fig. 7. The cells have scanty cytoplasm and show an extreme degree of flattening. H. & E., × 650.

FIG. 9.—High-power view of an adenoma which has been reactivated by 4 weeks of thiourea treatment after 3 weeks of treatment with thyroid digest. The cells are tall columnar, comparable with those of Fig. 2, and contrast strikingly with those of Fig. 6 and 8. There has been a partial resorption of colloid. H. & E., × 650.

FIG. 10.—Adenocarcinoma in a rat which received thyroid digest for 3 weeks before death. On the left are darkly staining adenocarcinoma cells. By comparison with Fig. 4 these cells are seen to be markedly involuted. The acinar spaces contain colloid but are not distended. On the right is a nodule composed of pale stained cells showing no acinus formation. This nodule is considered to be a foetal adenoma. H. & E., × 650.

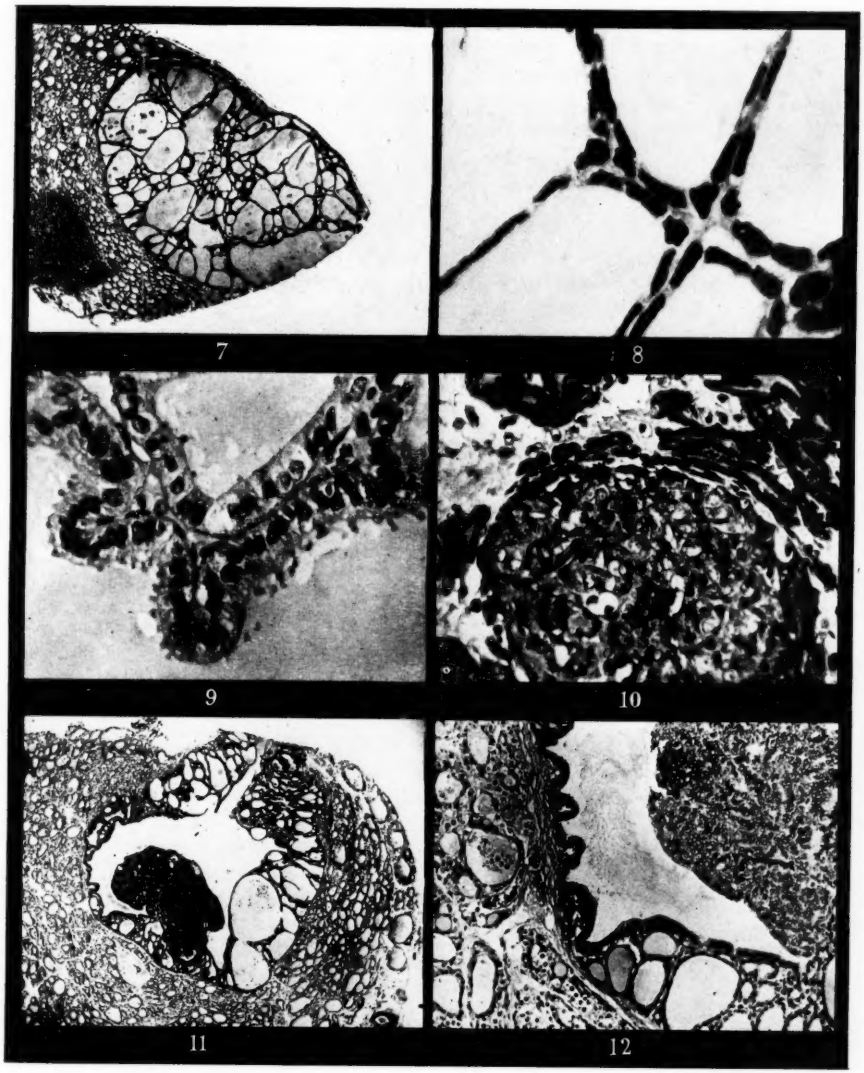
FIG. 11.—Cystic adenoma in thyroid of a rat after 17 months' thiourea treatment. A foetal adenoma forms a solid cellular growth projecting from the wall of the cyst. H. & E., × 30.

FIG. 12.—Part of Fig. 11 at higher magnification. The pale stained cells of the foetal adenoma in the top left-hand corner are arranged in cords. The wall of the cyst is composed of tall columnar epithelium, which is deeply stained and quite distinct from the cells of the foetal adenoma. H. & E., × 144.



Purves and Griesbach.

UNIV. OF MICH.


7
8
9
10
11
12

DISCUSSION.

The production of thyroid adenomata by the administration of thiourea is not unexpected, in view of the previous finding of thyroid adenomata produced by rape seed administration and the production of adenomata by allylthiourea administration reported by Bielschowsky (1945). In contrast to the rapid appearance of the thyroid tumours in rats treated simultaneously with acetamidofluorene and allylthiourea, as reported by Bielschowsky (1944), under the influence of the goitrogen alone thyroid tumours appeared only after a considerable delay. Once the tumours appear they apparently grow rapidly, and it therefore seems that tumours are initiated only after many months of treatment with the goitrogenic agent. In those animals which were treated for one year or more thyroid tumours were found in twenty-two out of twenty-five, giving an incidence of 88 per cent. In our experiments there was no certain indication of any difference in susceptibility of the two sexes or of the two strains which were tested.

Of the twenty-two animals which had thyroid tumours, seven showed tumours classifiable as malignant on histological appearances, giving an incidence of 31 per cent. The first of these malignant tumours was observed after 20 months. In the thirteen animals which were treated with thiourea for 20 months or more there were seven malignant tumours, an incidence of 54 per cent. The fact that the malignant tumours were always relatively large and in most cases were associated with smaller tumours of benign appearance situated in the other thyroid lobe leads to the conclusion that the early adenomata have taken on a malignant appearance under continued stimulation. However, there was nowhere any suggestion that a small nodule of malignant growth had appeared in a larger benign tumour. The change to the malignant appearance seems to be the result of an evolution in the growth of the whole tumour. The fact that Wegelin (1927) and Hellwig (1935) observed similar tumours in the thyroids of rats on iodine-deficient diets suggests that the tumours produced by goitrogenic agents are not the result of any carcinogenic action of the thioureas, but are the result of long-continued intense stimulation by the thyrotropic hormone. The fact that lung metastases were observed in some of our rats is not proof that these thiourea-induced tumours are more malignant than those resulting from iodine deficiency, since such lung metastases may easily be overlooked. Moreover, the fact that no neoplasia-inducing effects were observed in tissues other than the thyroid after this long period of thiourea administration is evidence against any direct carcinogenic action of the thiourea.

In three of the thyroid tumours observed in this series of experiments there were structures present which we have called foetal adenomata. These foetal adenomata have clearly arisen in the interior of the pre-existing adenoma or adenocarcinoma. In two cases the parent tumour was classified as adenocarcinoma. The interesting thing is that by secondary change a tumour of benign behaviour has arisen from one of malignant character. The association of foetal adenoma with malignant thyroid tumour in human thyroids has led some pathologists to the view that foetal adenoma is potentially malignant and may give rise to adenocarcinoma. Our observations of these early tumours in the rat suggests that the adenocarcinoma may well have antedated the foetal adenoma. The preconceived idea that benign neoplasms commonly give rise

to malignant growths by secondary changes needs careful consideration, since these studies suggest that, even where benign and malignant structures co-exist, it is by no means certain that the malignant structure has been derived from the benign. In these thyroid tumours there was never any evidence of a more malignant structure arising inside a pre-existing benign structure. In some tumours which were mainly follicular in structure there were areas where the cells were arranged in cords and trabecules without acinar formation. The idea that these areas might be a more malignant variation of the primary tumour is, however, negatived by the evidence of the metastases in the lung, where well-formed acini were found.

The thyroid neoplasms described in this paper were all responsive to fluctuations in the level of thyrotropic hormone. Indeed, in the case of the benign adenomata, colloid accumulation after administration of thyroid substance was much more marked than in the surrounding normal thyroid tissue. These involuted adenomata can be reactivated, and respond with a prompt cellular hypertrophy, though the resorption of the colloid is much slower than in the normal thyroid. This readiness to accumulate colloid and reluctance to discharge it is related to the tendency noted by Bielschowsky (1944, 1945) for these adenomata to contain even under conditions of stimulation a certain amount of colloid in contrast to the normal hyperplastic thyroid where colloid is almost entirely absent. This effect may be related to the large size of most of the acinar spaces, which results in the ratio of cellular surface to acinar volume being relatively less in the adenomata. The supply of proteolytic enzymes by which the solution of the colloid is effected is therefore less in the adenomata.

The " foetal adenoma " type of tissue did not show any reaction after thyroid administration. The cell nuclei were large and pale, the cytoplasm was well defined and there was no colloid accumulation.

The three tumours of carcinomatous type which had not been under thyrotropic stimulation for 21 days showed a definite epithelial change towards regression. The nuclei were pycnotic and showed the signs of mutual compression. The cytoplasm was hardly recognizable. In parts with trabecular configuration there was no colloid storage visible, but where a more acinar structure prevailed large colloid-filled vesicles were present. It is evident that malignant behaviour in thyroid tumours is not necessarily accompanied by escape from thyrotropic control. It may therefore be possible to influence the course of malignant thyroid disease in human beings by the therapeutic administration of desiccated thyroid.

SUMMARY.

1. Prolonged administration of thiourea to rats caused the formation of thyroid adenomata. In the second year of thiourea treatment 88 per cent of the animals showed adenoma formation. No significant sex difference or strain difference in susceptibility was observed.

2. After 20 months of treatment seven out of thirteen of these tumours showed the histological appearance of adenocarcinoma. In all seven cases invasion of the blood-vessel walls was visible. In two of these cases lung metastases of thyroid tissue were found.

3. In three thyroid tumours, two of which were definitely malignant, solid masses of cells were found which resembled the " foetal adenoma " of human

pathology. These growths showed benign behaviour. It is considered that these growths were derived from the cancerous tissue.

4. The thyroid adenomata observed in these experiments retained in full their ability to respond to fluctuations in thyrotropic stimulation. Inhibition of pituitary thyrotropic secretion was followed by prompt cell involution and marked colloid storage. When the thyrotropic stimulation was resumed there was a prompt cellular hypertrophy and signs of colloid resorption. The adenocarcinomata also showed full cell involution after withdrawal of thyrotropic hormone and stored colloid in such parts as had an acinar structure. In those parts showing only a trabecular appearance, withdrawal of thyrotropic hormone caused cell involution without producing colloid storage.

REFERENCES.

BIELSCHOWSKY, F.—(1944) *Brit. J. exp. Path.*, **25**, 90.—(1945) *Ibid.*, **26**, 270.
GRIESBACH, W. E., KENNEDY, T. H., AND PURVES, H. D.—(1945) *Ibid.*, **26**, 18.
GRIESBACH, W. E., AND PURVES, H. D.—(1945) *Ibid.*, **26**, 13.
HELLWIG, C. A.—(1935) *Amer. J. Cancer*, **23**, 550.
PURVES, H. D., AND GRIESBACH, W. E.—(1946) *Brit. J. exp. Path.*, **27**, 294.
WEGELIN, C.—(1927) *Schweiz. med. Wchnschr.*, **8**, 848.

INVESTIGATION INTO THE PRODUCTION OF BACTERIOSTATIC SUBSTANCES BY FUNGI. PRELIMINARY EXAMINATION OF THE SIXTH 100 SPECIES, MORE BASIDIOMYCETES OF THE WOOD-DESTROYING TYPE.

W. H. WILKINS.

From the Mycology Laboratory, University Department of Botany, Oxford.

Received for publication January 9, 1947.

THE present paper, an extension of the series which has appeared from this Laboratory since 1942, is a continuation of the work on basidiomycetes. It follows directly the line of its predecessor (referred to subsequently as "the previous paper") on "the fifth 100 species" (Wilkins, 1946*a*), and deals in similar manner with another hundred of the same sort of fungi. Further papers on basidiomycetes, the group on which work is at present concentrated, are in course of preparation.

As in the previous paper, the cultures were obtained from the Forest Products Research Laboratory (acknowledgments as before,) and, for the purposes of strain identification, the Laboratory Number is given to each species.

Each fungus was grown on malt and potato dextrose agar, and tested by the "strip" method (Wilkins and Harris, 1944*b*) against *Bacterium coli* and *Staphylococcus aureus*. In view of previous experience, the positive or negative result of this test was taken as sufficient evidence that the fungus did or did not produce

antibacterial substances, and only those that gave a positive strip result were further investigated. This differed from the technique of the previous paper, but resulted in a saving of time without any appreciable loss of accuracy. The strip-positive fungi were then grown on 10 ml. of liquid malt and potato dextrose, and the metabolism solutions were tested every five days against the same two representative types of bacteria by the " hole " method commonly used in this Laboratory (Wilkins and Harris, 1943*b*).

The results, which are listed below, are arranged in three classes : (1) The " strongly positive," i.e. those which, when tested by the hole method, gave an area of inhibition of 20 mm. or more in diameter, (2) the " weakly positive," which gave an inhibition area of between 15 and 20 mm., and (3) the " negative," being those which gave a negative result in the initial strip test. The strongly positive, in that they are regarded as the most promising, are listed in greater detail. In certain of the results listed as positive the zone of inhibition was not entirely clear of bacteria. This incomplete inhibition is apparently due sometimes to the medium and sometimes to the bacterium. The phenomenon has been noted by other investigators. In connection with the present work it has little direct significance, as no fungus is listed as positive unless at least one test gave a perfectly clear zone.

LIST OF FUNGI EXAMINED.

Strongly Positive.

	Strip.			Liquid.		
	Time in days.	Malt.	Potato dextrose.	Time in days.	Malt.	Potato dextrose.
Daedalea unicolor (Bull.) Fr. 110	7	0·16	0·13	20/10	0·27	0·21
Fomes officinalis Fr. 81 135	16	13·14	16·16	20/25	20·23	21·21
Irpex destruens Petch. 135	24	0·21	0·17	5	0·22	0·31
Lenzites saepiaria (Wulf.) Fr. 10A	16	14·21	0·18	25/20	22·26	17·20
L. thermophila Falck. 75	11	0·11	0·15	25/15	18·21	0·18
Polyporus borealis (Wahl.) Fr. 101	11	17·27	7·17	15	..	0·20
P. obtusus Berk. 198	8	13·30	13·24	10	0·20	0·18
P. spumeus v. *mongolica* Murash. 166	8	..	10·18	10/30	0·17	0·26
Poria crassa Karst. 163	8	8·14	0·15	20	19·21	20·19
P. hymenocystis B. & Br. 234	5	6·6	6·10	5/10	15·22	0·16
P. vaporaria Fr. sensu Bres. 280	5	8·8	8·8	15/30	20·19	24·24
Stereum rameale Schw. 265	7	0·20	0·19	20/10	17·32	0·31
Trametes lilacino-gilva (Berk.) Lloyd 172A	8	7·6	0·11	20	0·25	0·20
T. serialis Fr. 107B	6	10·11	8·8	20	17·18	0·21

Weakly Positive.

Coniophora cerebella Pers. 11B and 11D ; *Fomes fomentarius* (Linn.) Fr. 79F ; *F. igniarius* (Linn.) Fr. 97D ; *F. melanosporus* Mont. 283 ; *F. pinicola* (Sw.) Cke. 98 and 98A ; *F. roseus* (A. & S.) Fr. 140 ; *Hydnum erinaceum* (Bull.) Fr. 277 ; *Lentinus cochleatus* (Pers.) Fr. 171A ; *L. lepideus* Fr. 7 ; *Lenzites bicolor* Falck, 138 ; *L. saepiaria* (Wulf) Fr. 10C ; *Merulius molluscus* Fr. 94A ; *M. himantioides* Fr. 233 ; *M. sylvester* Falck, 72 ; *Pholiota lucifera* (Lasch.) Fr. 113 ; *P. spectabilis* Fr. 126 ; *Pleurotus palmatus* (Bull.) Quél. 170 ; *Polyporus balsameus* Peck, 114 ; *P. benzoinus* (Wahl.) Fr. 137A ; *P. Berkleyi* Fr. 100 ; *P. durus* Jungh, 205 ; *P. giganteus* (Pers.) Fr. 93 ; *P. orientalis* Lloyd, 146 ; *P. rhipidium* Berk. 260 ; *P. Schweinitzii* Fr. 102A ; *P. vulpinus* Fr. 192 ; *Poria subacida* Peck, 104 and 104A ; *P. tsugina* (Murr.) Sacc. & Trott. 194 ; *P. vaporaria* Pers. 14A ; *Stereum*

fasciatum (Schw.) Fr. 105 ; *S. frustulosum* Fr. 83 and 83A ; *S. lobatum* Fr. 263 ; *S. purpureum* Fr. 88 ; *S. subpileatum* B. & C. 271 ; *S. vellereum* Berk. 269 ; *Trametes carnea* Nees & Fr. 106 ; *T. cervina* (Schw.) Bres. 168 ; *T. serialis* Fr. 107C ; *Ustulina zonata* (Lév.) Sacc. 95A.

Negative by Strip Test.

Daedalea biennus (Bull.) Quél. 84C ; *Fomes Clelandi* Lloyd, 259 ; *F. conchatus* (Pers.) Fr. 142 ; *F. igniarius* (Linn.) Fr. 97 ; *F. lividus* Kalchbr. 293 ; *F. rubustus* Karst. 139 ; *F. vinosus* Berk. 143 ; *Ganoderma resinaceum* Boud. 144 ; *Lentinus praerigidus* Berk. 287 ; *Lenzites abietina* Bull. 77 ; *L. flaccida* Fr. 76 ; *L. repanda* (Mont.) Fr. 296 ; *Merulius corium* Fr. 30B ; *Peniophora gigantea* Fr. 175 ; *Pholiota squarrosa* (Müll.) Fr. 90 ; *Pleurotus sapidus* Schulz. 167 ; *P. ulmarius* Bull. 99A ; *Polyporus adiposus* B. & Br. 279 ; *P. anceps* Peck, 70A ; *P. arcularis* (Batsch.) Fr. 197 ; *P. borealis* (Wahl.) Fr. 101A ; *P. brumalis* (Pers.) Fr. 174A ; *P. circinatus* Fr. 115 ; *P. croceus* (Pers.) Fr. 145 ; *P. cuticularis* (Bull.) Fr. 278 ; *P. dryadeus* (Pers.) Fr. 96 ; *P. frondosus* Fr. 117A ; *P. gilvus* Schw. 133 ; *P. graveolens* Schw. 118 ; *P. rutilans* (Pers.) Fr. 188C ; *P. shorea* Wakf. 281 ; *P. squamosus* (Huds.) Fr. 103A ; *P. suaveolens* (Linn.) Fr. 121B ; *P. Rickii* (Pat.) Sacc. & Trott. 289 ; *P. velutinus* Fr. 229 ; *Poria Andersonii* (Ell. & Ev.) Neuman 288 ; *P. laevigata* Fr. 147 ; *P. Weirii* (Murr.) Sacc. and Trott. 286 ; *Stereum Murrayi* (B. & C.) Burt, 239 ; *S. necator* Viala, 268 ; *Trametes incerta* (Currey) Cke. 282 ; *T. mollis* (Sommerf.) Fr. 154 ; *Ustulina vulgaris* Fr. 95.

The above results are essentially comparable with those of the previous paper. They are an addition to the gradually accumulating detail, from which it is hoped to be able to make generalizations and to determine lines of investigation which will lead to understanding of the "strain variation" problem, which is, at present, a serious handicap to progress.

The results, published in a comprehensive paper by Robbins, Hervey, Ross, Ma and Robbins (1945), are still being examined in detail and will be discussed in a later paper. Since the previous paper was sent to press, papers by other investigators dealing with the antibacterial properties of the larger basidiomycetes have appeared. The following are particularly interesting in that they are comparable in many respects with some of the writer's earlier work on this group of fungi.

Mathieson (1946) examined 230 species of basidiomycetes collected in Victoria, Australia. She extracted sporophore "juice" as previously done by Wilkins and Harris (1944*e*) and Wilkins (1946*b*), and tested this for antibacterial activity against *Staph. aureus* and *Bact. coli*, using the "hole" method of Wilkins and Harris (1943*b*). Mathieson's list has 74 species in common with our list, and of these there is agreement in 52 instances. Considering the variation between strains of the same species already stressed by us and confirmed by Mathieson, this can be regarded as a reasonable degree of agreement. Of 5 species of *Psalliota* tested she found only one (unidentified) to be positive. As a result of testing 11 species of that genus I also found only one species to be positive.* This was *P. xanthoderma* and its varieties, and from Mathieson's remarks and from evidence given below, I suggest that her unidentified species was *P. xanthoderma* or a variety of this species.

* The *Psalliota arvensis* given in the list by Wilkins and Harris, 1944*d*, Table I, top of p. 167, as positive was, I think, a misidentification. It is more probably *P. xanthoderma*.

Atkinson (1946*a*) wrote a preliminary note on an investigation into the antibacterial activity of certain higher fungi. She developed this in a more detailed paper (1946*b*). She examined some 200 species of "toadstools and mushrooms" collected in various parts of S. Australia. She extracted the sporophore juice as done by Wilkins and Harris and by Mathieson, but tested for antibacterial activity by the Heatley method (1944). She found that any active extract inhibited the growth of *Staph. aureus*, but only two species, *Cortinarius rotundisporus* and *Psalliota xanthoderma*, also inhibited the growth of the Gram— *Salm. typhi* and the acid-fast *Mycobacterium phlei*. The *Psalliota* showed an equally strong reaction against both the Gram + and the Gram — organisms. In addition to the above bacteria, which were used for qualitative tests, the crude extracts of the *Cortinarius* produced strong inhibition against *Bact. coli*, *B. subtilis*, *C. diphtheriae mitis*, *N. catarrhalis*, and a Group A streptococcus. The *Psalliota* strongly inhibited *Bact. coli*, *B. subtilis*, and a Group B streptococcus. It also reacted against the vole tubercle bacillus, and was active against a staphylococcus which was relatively insensitive to penicillin. Neither species had any inhibitory effects on *Ps. pyocyanea*. Experimental evidence tends to the view that the two fungi produced different antibacterial substances. The activity of the substances was not affected by the addition of 10 per cent serum *in vitro*. Tests in mice of crude or partially purified substances indicated a low degree of toxicity. Isolation and purification of the antibacterial substances is not yet satisfactory.

Atkinson (1946*b*) states that whatever the locality from which the *P. xanthoderma* was obtained, it was always positive. I can confirm that this is also true of species collected in this country. This, though perhaps not unique, is exceptional and, in conjunction with the characteristics described by Atkinson, suggests that this fungus merits further investigation.

My grateful thanks are due to the Medical Research Council for a grant enabling me to employ research assistance and, in connection with the present work, I am particularly appreciative of the help given by Miss G. M. Trevaldwyn, M.A., and Miss J. W. Hawkes, B.Sc.

REFERENCES.

Atkinson, Nancy.—(1946*a*) *Nature*, **157**, 441.—(1946*b*) *Austral. J. exp. Biol.*, **24**, 169.
Heatley, N. G.—(1944) *Biochem. J.*, **38**, 61.
Mathieson, Jean.—(1946) *Austral. J. exp. Biol.*, **24**, 57.
Robbins, W. J., Hervey, A., Ross, W. D., Ma, R., and Robbins, W. C.—(1945) *Bull. Torrey bot. Cl.*, **72**, 165.
Wilkins, W. H.—(1945) *Trans. Brit. mycol. Soc.*, **28**, 110.—(1946*a*) *Brit. J. exp. Path.*, **27**, 140.—(1946*b*) *Ann. appl. Biol.*, **33**, 188.
Idem and Harris, G. C. M.—(1943*b*) *Brit. J. exp. Path.*, **24**, 141.—(1944*b*) *Nature*, **154**, 578.—(1944*d*) *Ann. appl. Biol.*, **21**, 161.—(1944*e*) *Trans. Brit. mycol. Soc.*, **27**, 113.

THE ROLE OF COAGULASE IN STAPHYLOCOCCAL INFECTIONS.

WILSON SMITH, J. H. HALE AND M. M. SMITH.

From the Departments of Bacteriology of the University of Sheffield and University College Hospital Medical School, London.

Received for publication January 11, 1947.

THE pathogenicity of staphylococci exhibits certain features peculiar to this organism which have never been adequately explained, such as the tendency to the production of circumscribed local lesions, the inadequacy of the immune response to infection, the difficulty of artificial immunization, etc. Our interest in staphylocoagulase was aroused in the first instance because circumstantial evidence suggested that it might be a major factor in the initiation, and possibly also in the later development, of staphylococcal infections. More direct evidence in support of this view was obtained by a study of the influence of coagulase upon the phagocytosis of staphylococci *in vitro* (Hale and Smith, 1945). The present paper reports a further investigation of the problem by *in vivo* experiments. These, as we expected, have emphasized both the complexity of the processes involved in natural infection, and the artificial nature of experimentally induced infections of animal species which are normally non-susceptible. The results, however, whilst not minimizing the importance of other factors like alpha toxin, do show that the initial infectivity of staphylococci is largely dependent upon their power of coagulase production, so that without this power the other virulence factors are probably of little avail.

MATERIALS AND METHODS.

Strains of staphylococci.

The experiments are based upon the variability of different animal species in respect of plasma coagulability. In a previous paper we showed that most strains of *Staphylococcus pyogenes* fail to coagulate guinea-pig or mouse plasmas at 37° C. because of a deficiency of some activator substance in the blood of these species (Smith and Hale, 1944). Occasional strains, however, produce a coagulase which is effective against one or other of these activator-deficient plasmas (Hale and Smith, 1945). The routine testing of all available strains over a considerable period provided a number with the different qualities necessary for our purpose (Table I). In the text the unqualified designation of a strain as coagulase positive indicates that it possesses the normal coagulase activity of most pathogenic staphylococci against human, horse and rabbit plasmas; all strains listed in Table I, with the exception of strain AL, fall into this category. Coagulase G.P. + indicates additional activity against guinea-pig plasma; examples are strains 21, 2 and B. For a staphylococcus totally devoid of both coagulase activity and alpha toxinogenicity it was necessary to use an albus strain AL.

Alpha toxinogenicity was estimated by the sheep blood agar plate method of Bryce and Rountree (1936). The selected strains include strongly toxinogenic, weakly toxinogenic and non-toxinogenic staphylococci both with and without associated coagulase activity for guinea-pig plasma. It is important to bear in mind, however, that the failure of a staphylococcus to produce alpha toxin in plate cultures does not necessarily mean that no toxin will be formed during its growth in the animal body.

Experimental animals.

Most previous work on staphylococcal infectivity has been done with rabbits, but as all aureus strains tested by us have given positive coagulase reactions with rabbit plasma, this species has been of little use for our purpose. Guinea-pigs and mice have therefore been used throughout. The guinea-pig provides the ideal test animal for assessing the significance of coagulase activity in experimental infections, by direct comparisons of coagulase G.P. + and coagulase G.P. − strains, possessing equal alpha toxinogenicities of various grades. Similar comparisons could not be made with mice because this species can rarely be infected in the true sense, and unfortunately we have so far encountered only one staphylococcal strain possessing any coagulase activity for mouse plasma, and the activity in this case was of such low grade that only a very small loose clot was formed within four hours in the standard test. The mouse was therefore chiefly used to study the effects of a coagulable substrate upon the minimal lethal dose of staphylococci and on their survival within the tissues of the host.

TABLE I.—*The Coagulase Activities and Alpha Toxinogenicities of Staphylococcal Strains.*

Strain.	Coagulase activity on plasma of—				α toxin.
	Human.	Rabbit.	Mouse.	Guinea-pig.	
E	+	+	−	−	+
21	+	+	−	+	−
35	+	+	−	−	±
2	+	+	−	+	±
24	+	+	−	−	++
15	+	+	−	+	++
3	+	+	−	+	+
22	+	+	−	+	±
B	+	+	±	+	±
AL	−	−	−	−	−

CORRELATION OF COAGULASE ACTIVITY, TOXINOGENICITY AND VIRULENCE OF SELECTED STRAINS.

Intratesticular inoculation of guinea-pigs.

Six staphylococcal strains with various coagulase and toxinogenic activities were compared by the intratesticular inoculation of guinea-pigs, a group of three animals being used for each strain. Each inoculation consisted of 0·2 ml. of an

18-hour digest broth culture. The guinea-pigs were killed at 3, 4 and 6 days after infection. In a second similar experiment saline suspensions of the staphylococci were prepared from cultures whose content of viable organisms had been determined; a standard dose of 76 million viable organisms was given in 0·2 ml. volume and the animals were all killed on the 4th day. The results of the two experiments are summarized together in Table II.

TABLE II.—*The Infectivity of Different Staphylococcal Strains for Guinea-pig Testis.*

Strain.	G.P. coag.	α toxin.	Degree of testis infection when killed. Day.						Average score.
			3.	4.	4.	4.	4.	6.	
15	+	++	5	5	5	5	5	3	4·7
21	+	—	4	5	2	5	3	3	3·7
24	—	++	2	0	1	0	3	5	1·8
2	+	±	5	4	3	4	5	2	3·8
35	—	±	1	1	4	1	2	0	1·5
Al	—	—	0	0	0	0	1	0	0·2

Figures represent degrees of testicular involvement:
0. Nil.
1. Engorgement.
2. Very small abscess.
3. Medium-sized abscess.
4. Large abscess, multiple abscesses or abscess with necrosis and adhesions.
5. Almost complete necrosis.

In each group of six guinea-pigs, except the AL group, there was considerable individual variation of response. Each animal was therefore given a score from 0 to 5 as a measure of the extent of infection and degree of tissue damage observed at autopsy, so that the average scores shown in the last column of Table II are indicative of the relative virulence of the various strains. The average scores for each of the three coagulase G.P. + strains are significantly higher than those for the three G.P. — strains irrespective of toxinogenicity. It is true that the two toxin-producing staphylococci devoid of coagulase activity, strains 24 and 35, caused lesions in the majority of inoculated testes with extensive damage in two cases, but the results with strain 21, coagulase G.P. +, toxinogenicity —, suggest that the influence of coagulase activity far outweighs that of toxinogenicity. *Staph.* 15, which possesses both types of activity in high degree, produced almost complete testicular necrosis in five of six animals. The albus strain on the other hand produced no detectable response in five, and merely some vascular engorgement in one.

Intradermal inoculation of guinea-pigs.

The same six staphylococcal strains were compared by the intradermal inoculation of groups of three guinea-pigs with 0·2 ml. of 18-hour digest broth cultures adjusted to equal opacities. The score method of assessing results was not applicable to this experiment because of time variations in both the development and the subsequent regression of lesions, due in all probability to anatomical differ-

ences and the difficulty of making inoculations at a uniform depth in the skin. Each of the three coagulase G.P. + staphylococci produced purulent and necrotic lesions, but the results differed from those of the intratesticular experiment in that lesions of comparable severity were also caused by the highly toxinogenic coagulase G.P. —, strain 24. The remaining two strains, 35 and AL, were innocuous.

In order to eliminate as far as possible the factor of individual variability of response five tests were carried out, in each of which a coagulase G.P. + and a coagulase G.P. — strain were compared by intradermal inoculations into the opposite flanks of a guinea-pig. Two new strains were included in this experiment. The results, summarized in Table III, confirm those of the earlier experiment. All G.P. coagulase producers and also the highly toxinogenic strain 24 produced progressive skin lesions, characterized by necrosis, pus formation and ulceration. Whether the anomalous behaviour of staph. 24 is due to its high degree of alpha toxinogenicity or to some other unknown factor is uncertain, but

TABLE III.—*The Infectivity of Different Staphylococcal Strains for Guinea-pig Skin.*

Expt.	Strains.	Dose.	G.P. coag.	α toxin.	Result.
1	E	0·2 ml. cult.	—	+	Slight thickening; small bead pus expressed 3rd day.
	3	0·2 „	+	+	Progressive development of abscess with ulceration.
2	35	80×10^6 orgs.	—	±	Trivial thickening disappeared by 3rd day.
	2	80×10^6 „	+	±	Inflammatory nodule; no regression until 7th day.
3	35	80×10^6 orgs.	—	±	Trivial thickening disappeared by 3rd day.
	2	80×10^6 „	+	±	Inflammatory nodule; ulceration with discharge of pus 4th day.
4	24	0·2 ml. cult.	—	++	Inflammatory nodule; discharge of pus; punched-out ulcer 6th day.
	21	0·2 „	+	—	Inflammatory nodule; discharge of pus; progressive to 5th day.
5	24	0·2 ml. cult.	—	++	Inflammatory nodule; discharge of pus 3rd day; no regression till 6th day.
	21	0·2 „	+	—	Inflammatory nodule; discharge of pus 3rd day; no regression till 6th day.

it is probably significant that of the two other coagulase G.P. — strains the moderately toxinogenic staph. E showed slight pyogenicity whereas the very weakly toxinogenic staph. 35 was without any such effect. That a relatively large inoculation of a toxinogenic organism into a dense tissue like skin should produce necrosis is not surprising ; what is more difficult to explain is the similar effect produced by coagulase G.P. + organisms which do not produce alpha toxin under standard conditions *in vitro*, for coagulase itself does not cause damage to tissues. Such strains must either form alpha toxin *in vivo* or produce some other unknown toxin.

Intracardiac and intravenous inoculations of guinea-pigs.

The minimal lethal doses of six strains were estimated by intracardiac inoculations of guinea-pigs. Groups of three animals were given doses ranging from 2 ml. to 0·2 ml. of 18-hour digest broth cultures, matched to an opacity standard, and the viable organisms in each culture were enumerated by plate counts. End points were taken as the smallest number of viable cocci to kill two out of three guinea-pigs within seven days, at which time all survivors were killed and examined for staphylococcal lesions. The results summarized in Table IV show a striking correlation between virulence and coagulase activity for guinea-pig plasma. The M.L.D.'s of the non-coagulase producers are from four to eight times greater than those of the coagulase positive strains. No such correlation exists between virulence and alpha toxinogenicity ; highly, weakly and non-toxinogenic strains fall into both the virulent and avirulent groups. Moreover, the M.L.D. of the highly toxinogenic but coagulase G.P. negative staph. 24 is eight times larger than the non-toxinogenic coagulase G.P. positive staph. 21. The lesions found in the internal organs of survivors indicated that invasiveness is likewise dependent upon power of coagulase production. The trivial lesions in survivors of the coagulase negative group were confined to the kidneys and, in view of the relatively enormous doses of cocci inoculated directly into the circulation, can scarcely be regarded as infections in the true sense. The majority of survivors in the coagulase positive group, on the other hand, showed multiple abscesses of various sizes not only in the kidneys, but also in other sites such as liver, spleen and heart muscle.

TABLE IV.—*The Infectivity of Different Staphylococcal Strains Inoculated Intracardially in Guinea-pigs.*

Strain.	Guinea-pig coagulase.	α Toxin.	M.L.D. (millions).	Dosage range tested (millions).	Lesions in survivors.
24	—	++	1740	3480–348	+
35	—	±	1750	>1750–440	±
AL	—	—	1500	>1500–750	±
15	+	++	366	366–72	++
2	+	±	340	680–34	++
21	+	—	217	217–43	++

Lesions in survivors :—

++ Majority of survivors had multiple abscesses in internal organs.
+ Majority of survivors had one or two small abscesses only.
± Majority of survivors had no lesions, or one or two minute foci in kidneys.

A further experiment in which strains 2 and 35 were compared by intravenous inoculation *via* the internal saphena vein fully confirmed the importance of coagulase activity as a factor in the invasiveness of staphylococci. Graded doses of cocci in saline suspension were given in a constant volume of 0·5 ml. and the animals were killed on the 5th day. In view of the difference in M.L.D.'s established earlier the dose range employed for staph. 35 was from 600 to 1800 million cocci, and for staph. 2 was from 200 to 800 million. The strains failed to kill at the expected doses, probably because leakage of some of the inoculum into the loose cellular tissue around the vein occured in most cases. Thus, apart from one non-specific death on the day following inoculation, only two guinea-pigs died, one in each series. The fatal case in the *Staph.* 35 series was found to be pregnant and showed no generalized staphylococcal lesions ; that in the *Staph.* 2 series had multiple abscesses in lung, liver and both kidneys. Of the survivors only one of eight guinea-pigs inoculated with strain 35 had internal lesions in the form of a very small septic infarct of each kidney, but with strain 2 no less than seven out of eleven animals had abscesses either in kidneys or liver or both organs, some of which were more than 1 cm. in diameter.

When it is remembered that in both the intracardiac and intravenous inoculation experiments doses were adjusted to be heavily in favour of the coagulase negative strains, it is difficult to escape the conclusion that the determinant factor in virulence and invasiveness is power of forming a coagulase effective against the plasma of the host recipient.

Intravenous inoculation of mice.

If this conclusion is valid the six strains should give no similar sharp division into virulent and avirulent groups when tested in mice, because none of them forms a coagulase effective against mouse plasma. This was in fact found to be the case in a number of experiments designed on the same lines as the guinea-pig tests. The M.L.D. of each strain varied from experiment to experiment because of variability in the sizes and weights of different batches of mice, but in any one experiment on a uniform batch of animals the M.L.D.'s of different strains were usually equal, and the occasional discrepancies could never be correlated with either G.P. coagulase activity or with alpha toxinogenicity, nor could the same discrepancy be obtained in repeat tests. Relatively enormous doses were required to kill mice, never less than 200 million viable cocci, and there is little doubt that deaths were due to the non-specific endotoxins of the inoculated organisms rather than progressive infection. Lesions in survivors from large doses were usually small septic infarcts, chiefly in the kidneys, where apparently the cocci are mechanically filtered out of the circulation.

THE EFFECT OF A COAGULABLE MENSTRUUM ON THE VIRULENCE OF STAPHYLOCOCCI.

The virulence of plasma-treated staphylococci for mice.

In a previous paper we suggested that the correlation between the pathogenicity and coagulase production of staphylococci was due chiefly to the protection against phagocytosis afforded to the microbes by the barrier of fibrin formed from the plasma and tissue fluids of the host (Hale and Smith, 1945). We attempted to confer such a protective barrier on staphylococci in order to

enhance their virulence for mice. In a series of nineteen experiments groups of from ten to twenty mice were inoculated intraperitoneally or intravenously with a measured dose of a coagulase positive staphylococcus mixed with human or rabbit plasma, control mice receiving the same dose together with human serum, rabbit serum or guinea-pig plasma. Before inoculation the mixtures were allowed to stand either at room temperature for half an hour or in a water bath at 37° C. for a suitable predetermined time, in the hope that during this interval coagulase action would have begun at the surfaces of the organisms, thus lessening the chances of all plasma being washed away within the peritoneal cavity of the mouse. The results are summarized in Table V.

TABLE V.—*The Increased Virulence of Staphylococci for Mice when Inoculated with a Coagulable Plasma.*

Expt. No.	Staphylococci mixed with—	Mice. No.	Mice. Died.
1 to 3	Human plasma	43	31
	Guinea-pig plasma	43	20
4 to 8	Human plasma	54	43
	,, serum	54	23
9 to 19	Rabbit plasma	144	85
	,, serum	144	62
Totals	Coagulable plasmas	241	159
	Control fluids	241	105

The summed mortality rates indicate that a coagulable menstruum enhances the virulence of the organisms. This effect, however, was not obtained in every individual experiment; although very striking in many experiments, there were others in which the mortality rates of plasma and control groups were approximately equal. This discrepancy does not invalidate the significance of the results as a whole. The introduction of certain foreign proteins into the peritoneal cavity inhibits the phagocytosis of any particulate matter present by affecting adversely mobilization of the phagocytic cells. Different samples of plasma or serum vary greatly in potency in this respect, so that any staphylococcal strain tends to show wide variations of virulence from experiment to experiment when such fluids are incorporated in the inocula. Thus demonstration of the coagulase effect requires a nice adjustment of the infecting dose of organism, and will be overshadowed by the non-specific protein effect in any experiments in which the critical dose has not been selected. If broth suspensions are used for the control batches of mice, mortality rates of over 50 per cent for plasma groups and *nil* for control groups can be obtained regularly, but the plasma group figures then represent the sum of the effects due to plasma treatment and inoculation of foreign protein. The foreign protein effect, unlike the specific plasma effect, can be demonstrated with non-coagulase producing organisms. In a number of experiments with *Streptococcus pyogenes*, suspensions in either human plasma or human serum proved much more virulent than suspensions in broth, but there

was no difference between the plasma and serum suspensions like those demonstrated with staphylococci.

The avirulence for mice of staphylococci suspended in saline or broth and the unpredictable behaviour of serum suspensions probably accounts for the fact that this organism has been little used in mouse chemotherapeutic tests. A number of preliminary experiments have shown the practicability of employing plasma suspensions of staphylococci for the purpose. Satisfactory end points can be obtained in M.L.D. titrations, and the prophylactic and therapeutic efficacy of penicillin readily demonstrated.

The fate of staphylococci injected into the peritoneal cavities of mice.

An attempt was made to study the course of events following intraperitoneal inoculation of mice with staphylococci suspended in coagulable and non-coagulable substrates. Mice were killed at intervals for the study of smears of peritoneal exudate, omentum, spleen and liver. The number of variables involved in experiments of this nature prevents any accurate quantitative analysis, and the time sequence varies from experiment to experiment within fairly wide limits; nevertheless the picture is sufficiently clear to indicate the significance of coagulase activity during the early phases of an infection. Following the inoculation of staphylococci in a non-coagulable menstruum there is an initial clearance of most of the organisms from the peritoneal cavity, due largely to their mechanical removal by the omentum. This occurs within the first hour before there is any appreciable cellular reaction or evidence of phagocytosis. Thereafter immigration of polymorphonuclear leucocytes occurs. The leucocytic response is very pronounced by two hours after the inoculation, at which time monocytes make their appearance. The cellular reaction increases for some hours, but quite soon monocytes preponderate. The number of free cocci is progressively reduced; at 24 hours they are again virtually absent from smears of peritoneal exudate, and from this time onwards intracellular cocci become increasingly difficult to find, although nomocytes may still be numerous for three or four days.

The course of events following the inoculation of a similar dose of the staphylococci in a coagulable plasma follows the same general pattern, but is modified by a striking increase of the number of organisms in the peritoneal exudate a few hours after the initial decrease. This is almost certainly due to multiplication of those cocci which have escaped mechanical clearance. The polymorphonuclear leucocyte response appears to be but little affected, but the monocyte response is delayed, and is less vigorous than with a non-coagulable menstruum. Likewise phagocytosis, especially by the monocytes, is less vigorous but more prolonged. Smears of peritoneal exudate taken 24 hours after inoculation show very large numbers of cocci, both free and intracellular. If the mouse is destined to survive the defences gain the upper hand and eventually all the cocci are ingested.

It seems probable, therefore, that the inhibition of phagocytosis due to the protective barrier of fibrin formed round the cocci as a result of coagulase activity, which is so readily demonstrable by *in vitro* experiments, is a most important factor in the initiation and establishment of infection. In the experiments discussed the protection conferred upon the staphylococci actually inoculated sufficed to allow considerable multiplication before the body defences were fully mobilized. It is reasonable to assume that in the presence of a constant supply of coagulable plasma many daughter cocci too would have the opportunity of

providing themselves with a protective barrier, so that a progressive infection would result. Attempts to provide a constant supply of coagulable plasma in the mouse by repeated intravenous and intraperitoneal injections were frustrated by the animals' intolerance to such treatment.

Plasma sensitization of guinea-pigs.

The intradermal inoculation of guinea-pigs with the coagulase G.P. — *Staph.* E results at most in a trivial reaction in spite of its alpha toxinogenicity (see Table III). Given subcutaneously without adjuvant the organism has even less effect unless relatively enormous doses are used. Experiments were carried out in which guinea-pigs were inoculated subcutaneously with 4 or 5 ml. of either human plasma or digest broth, and immediately thereafter with doses of *Staph.* E broth culture ranging from 0·1 ml. to 1 ml. into the same site. In all sixteen animals were used. The eight controls which received broth and culture developed oedematous swellings round the inoculation sites which subsided completely within the course of two or three days. There was no sign of infection except with the large doses of culture, which produced small superficial abscesses with central necrosis. Reactions in the eight guinea-pigs treated with plasma were in striking contrast. In every case a large oedematous, inflammatory swelling developed, followed by extensive abscess formation and necrosis. With the larger doses of the staphylococcus, ulceration ensued with the free discharge of pus, and the spread of infection along the flanks and to the mid-abdominal line necessitated early sacrifice of the animals. The same dose of human plasma, given without staphylococci, was without apparent effect apart from the transient swelling produced by the large inoculum.

Several attempts were also made to sensitize both guinea-pigs and mice by repeated inoculations of human plasma, given intravenously or intraperitoneally. In many cases such treated animals appeared to be more susceptible to staphylococcal infection than untreated controls, but undesirable effects from the multiple doses of foreign animal protein led us to abandon this type of experiment.

DISCUSSION.

The results of the experiments described leave little room for doubt that staphylocoagulase is an important factor in the pathogenicity of the organism. No claim is made that it is the only, or even the most important, factor. The extensive cellular necrosis of the characteristic focal lesions is evidence of toxic action in which coagulase can have no direct part ; present indications are that alpha toxin, leucocidin and possibly other tissue toxins, at present unknown, are responsible. Toxin, however, cannot be elaborated until the organism has gained a foothold in the host and begun to multiply, and we suggest that at the initial stage of natural infection with a small number of staphylococci coagulase production is absolutely essential if the infection is to progress. At later stages it may still aid the further multiplication of the organism in defiance of the ensuing phagocytic response, and may determine the outcome when infected emboli are transported to other sites. If this is so the close correlation which undoubtedly exists between the power to produce coagulase and the pathogenicity of staphylococci is understandable.

The use of species not normally susceptible to infection with the majority of

pathogenic strains of staphylococci may be open to criticism, but it allows analysis of the different factors concerned by methods which could not be applied to susceptible species. It might also be objected that the atypical strains, designated coagulase G.P. +, showed, in spite of their coagulase activity, little tendency to produce progressive infection in guinea-pigs unless very large doses were used. The coagulability of guinea-pig plasma by such strains, however, is of a lower order than that of rabbit or human plasma ; the clots take much longer to form and are usually of a looser character. In view of the rapidity of the phagocytic response, this time factor is probably of major importance.

In studying the effect of a coagulable menstruum on the fate of staphylococci injected into the peritoneal cavity of the mouse, it was clear that the protection conferred upon the cocci actually inoculated sufficed to allow considerable multiplication before the body defences were fully mobilized. It is reasonable to assume that in the presence of a constant supply of coagulable plasma, many daughter cocci too would have the opportunity of providing themselves with a protective barrier of fibrin. Much more critical tests could therefore be performed if guinea-pigs and mice could be converted into animals with rapidly coagulable plasma ; this might indeed be possible if coagulase activator could be obtained in concentrated and relatively pure form. Our attempts to obtain the same effect by injection of heterologous plasma into the circulation were frustrated by the rapid elimination of single doses of such foreign material and the intolerance of the animals to multiple inoculations.

At present there seems little prospect of conferring immunity against staphylococci by measures directed against their coagulase activity. Most workers agree that coagulase itself is non-antigenic, and that anticoagulase immune bodies are not produced in response to natural infection. Recently Lominski and Roberts (1946) have demonstrated an inhibition of coagulase activity by the majority of human sera tested, but the effect requires preliminary incubation of the coagulase serum mixtures and, in our view, is explicable more readily on the basis of thermal or enzymic destruction of the coagulase-activator complex than on an antigen-antibody combination. This hypothesis is not excluded by the fact that sera heated to 56° C. or 63° C. retain their inhibitory power, for we have found that activator is much more heat stable in serum than in the watery extracts of testis used in our earlier work. Non-antigenicity of coagulase in its natural state, however, does not preclude the possibility of conferring antigenicity by coupling with a carrier protein. Such considerations call urgently for the isolation and chemical definition of coagulase.

The discovery of strains of staphylococci which clot guinea-pig plasma and one strain with some degree of additional activity for mouse plasma incline us to the view, mentioned as a possibility in the first paper of this series, that the substance in serum which we called activator may be the prothrombin-like substrate, whilst coagulase serves as a kinase activator. In either case it becomes necessary to postulate strain differences in coagulases, and corresponding differences in the plasma constituents of diverse animal species.

SUMMARY.

1. Some strains of *Staph. pyogenes* produce coagulase capable of clotting guinea-pig plasma. G.P. coagulase + staphylococci include highly, weakly and non-toxinogenic strains, as do also G.P. coagulase — organisms.

2. G.P. coagulase + strains are more virulent for guinea-pigs than G.P coagulase — strains, irrespective of their alpha toxinogenicity. This is shown by their lower M.L.D. and their greater infectivity when inoculated by various routes.

3. No such difference between strains can be demonstrated in mice. The virulence of staphylococci for mice, however, can be enhanced by suspending the organisms in a coagulable plasma. The fibrin which forms around the cocci protects them against the primary clearing mechanism of the host, and favours their multiplication and dissemination through the host tissues.

4. The experimental results suggest that coagulase is a major factor in the initiation and development of staphylococcal lesions, but that once the organisms have gained a foothold other factors like alpha toxin production come into play.

Acknowledgment.

One of us (M. M. S.) was in receipt of a part-time grant from the Medical Research Council, to whom we are also indebted for a grant towards the expenses of the work.

REFERENCES.

Bryce, L. M., and Rountree, P. M.—(1936) *J. Path. Bact.*, **43**, 173.
Hale, J. H., and Smith, W.—(1945) *Brit. J. exp. Path.*, **26**, 209.
Lominski, I., and Roberts, G. B. S.—(1946) *J. Path. Bact.*, **58**, 187.
Smith, W., and Hale, J. H.—(1944) *Brit. J. exp. Path.*, **25**, 101.